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Photoprotective role of beta -carotene and antioxidant enzymes: A shield against ultraviolet damage in Dunaliella bardawil

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PHOTOPROTECTIVE ROLE OF BETA-CAROTENE AND ANTIOXIDANT ENZYMES: A SHIELD AGAINST ULTRAVIOLET DAMAGE IN *DUNALIELLA BARDAWIL*

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DISSERTATION

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This dissertation has been examined and approved.

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DEDICATION

I would like to dedicate my dissertation to my husband, Kevin, and my parents. Thank you for the love and support you have provided throughout the many years of my graduate career. I truly appreciate all you have done to assist me in my endeavors...I could not have made it this far without you.

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ABSTRACT

PHOTOPROTECTIVE ROLE OF BETA-CAROTENE AND ANTIOXIDANT ENZYMES: A SHIELD AGAINST ULTRAVIOLET DAMAGE IN *DUNALIELLA BARDAWIL*

by

Andrea L. Grossman-White

University of New Hampshire, May, 2002

Photosynthetic and antioxidant responses following exposure to an array of irradiances and frequencies were examined and contrasted in two species of the unicellular green alga *Dunaliella*. Species selection was based on the ability of *Dunaliella bardawil* (UTEX 2538) to accumulate massive quantities of inter-thylakoid β-carotene during exposure to stress or high irradiances of photosynthetically active radiation (PAR: 400-700 nm) while *Dunaliella salina* (UTEX 200) lacks this ability. Cells were cultured in two irradiances (150 and 35 μmol photons $m^{-2} s^{-1}$), and then exposed to high red (wavelengths > 590 nm) or blue light (390-540 nm; 150 μmol photons $m^{-2} s^{-1}$), ultraviolet-A (320-400 nm) or ultraviolet-B (290-320 nm), independently, for 24 hour test periods. Ascorbate and glutathione concentrations, ascorbate peroxidase activity, photosynthetic oxygen evolution and fluorescence parameters were monitored following exposure to UV and various light treatments to assess changes in photosynthetic processes. In addition, polyacrylamide gel electrophoresis was utilized to quantify the number of catalase and superoxide dismutase isoenzymes present in *Dunaliella bardawil* and *Dunaliella*

salina, and to observe changes in catalase isozyme activity following exposure to different light regimes. There was no significant change in photosynthetic or antioxidant parameters following exposure to ultraviolet-B. Ultraviolet-A exposure significantly decreased photosynthetic capacity (>70% decrease in variable to maximum fluorescence and light limited to light saturated photosynthesis in low β-carotene cells) and resulted in 50% increases in ascorbate peroxidase activity and both ascorbate and glutathione concentrations. Exposure to UV-A resulted in decreased catalase activity and inactivation of related isozymes. The results suggest exposure to ultraviolet-A (but not ultraviolet-B) directly affects photosynthesis, observed as a loss of photosynthetic electron transport efficiency (and increased radical formation). *Dunaliella bardawil* was shown to possess three isoforms of the enzyme catalase while two isozymes were visualized for *Dunaliella salina.* Isozymes found in *Dunaliella bardawil* exhibited varying degrees of photosensitivity. It has been determined that the accumulated β-carotene in *Dunaliella bardawil* prevents UV-related photosynthetic damage through blue-light/ultraviolet-A absorption and is supported by trends observed for antioxidant enzyme responses.

INTRODUCTION

The formation of reactive oxygen intermediates (ROI's; or reactive oxygen species, ROS) in plant cells is a damaging process, both physically and physiologically. As a result, there has been selection of metabolic strategies that catalyze the breakdown of these potentially damaging molecules (Asada, 1999; Halliwell and Gutteridge, 1999). In nature, the formation of ROI's is often amplified by the interaction between high-energy radiation (sunlight) and metabolic processes within photosynthetic cells (Asada and Takahashi, 1987). The formation of the relatively stable oxidant hydrogen peroxide (H_2O_2) is an important aspect of cellular metabolism. Through metal-catalyzed reactions, highly reactive molecules may be formed from H_2O_2 which can lead to cellular damage (Halliwell and Gutteridge, 1999). Research has suggested that antioxidant scavenging responses to ROI formation within cells may be an important key to the prevention of cellular damage (Niyogi, 1999; Collen and Pederson, 1996; Malanga and Puntarulo, 1995; Foyer and Mullineaux, 1994; Asada and Takahashi, 1987).

Properties of solar radiation and absorption in photosynthesis

The nature of solar irradiance may be regarded in one of two ways, as an electromagnetic wave or an emission of particles, typically referred to as either quanta or photons. Visible radiation consists of wavelengths ranging from 400-700nm, while ultraviolet radiation ranges from 280-400nm. While there is an inverse

relationship between wavelength and energy, there is a direct relationship between frequency and energy, thus the blue to ultraviolet portion of the light spectrum possesses much greater potential energy than the red with regard to photosynthetic processes in plants (Heldt, 1997).

Light is absorbed and converted to chemical energy by oxygenic photosynthetic organisms from within the visible light spectrum; hence these wavelengths are typically referred to as photosynthetically active radiation. When light is absorbed by photosynthetic pigments there is a molecular conversion of that pigment from its ground state (lowest energy state) to an excited state resulting from electron movement to a higher energy orbital (Heldt, 1997). After trapping reactions, this energy is then passed through a series of reduction-oxidation along a series of electron carriers enabling sunlight to drive the process of photosynthesis and ultimately fix atmospheric carbon into a long-term energy storage product in the form of a carbohydrate (Halliwell and Gutteridge, 1999; Heldt, 1997).

In order to capture the photons produced by the sun, there are three types of photosynthetic pigments that have been identified: chlorophylls, phycobilins, and carotenoids. Although phycobilins are present in cyanobacteria and members of the algal group, rhodophyta, they are not present in higher plants or other classes of algae (Hall and Rao, 1994). Chlorophyll is considered the main pigment of photosynthetic light absorption and is an integral part of the reaction center structure (Heldt, 1997). There are several classes of chlorophylls, all of which share similar characteristics including a tetrapyrrole structure (named porphyrin), covalently bound Mg^{2+} in the center of the ring, and a long, branched hydrocarbon chain, called phytol rendering

the molecule extremely nonpolar (**Fig. 1**; Buchanan, et al., 2000; Heldt, 1997). The various classes of chlorophyll pigments are based on the structure of the phytol tail and the degree of saturation of the ring system (Buchanan, et al., 2000). Chlorophyll *a* is of particular importance as is it the central component of the photosynthetic reaction centers (PSI and PSII). As a result, the ratio of chlorophyll *a* to chlorophyll *b* in plants, while variable, is commonly about three to one (Heldt, 1997). Chlorophyll exhibits maximum absorbance at approximately 430nm (blue) and 680nm (red), reflecting most wavelengths between 500-600nm (commonly referred to as the "green window"; Heldt, 1997).

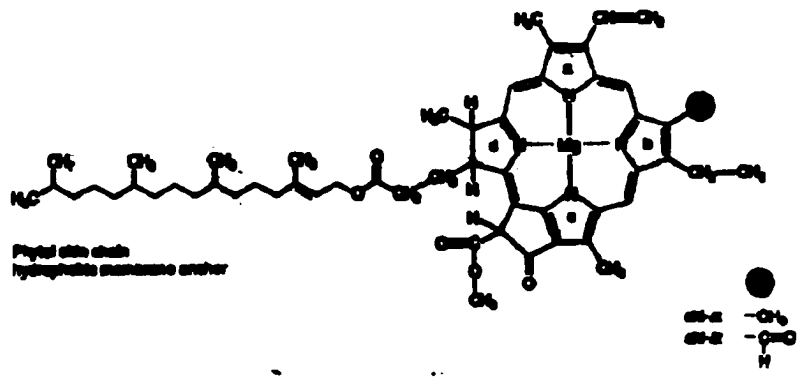

Fig. 1. Molecular structures of the photosynthetic pigments chlorophyll *a* and *b* (Heldt, 1997).

As previously described, in order for chlorophyll to absorb and utilize the energy from light, electrons must be excited to a higher energy level. When a single electron is raised to a higher energy orbital the molecule is said to be in either a singlet or triplet state (based on the spin direction). Heldt (1997) reports that as the number of double bonds increases within a molecule, the amount of energy required to produce

a first singlet state decreases. For this reason, chlorophyll requires exposure to red light only for the first singlet state to be obtained (due to the large number of double bonds present in the molecule).

Once chlorophyll has been excited to the first singlet state, it can return to the ground state in one of several ways. First, and most importantly to the process of photosynthesis, the chlorophyll molecule may transfer the excited electron to a nearby electron carrier, leaving behind a positively charged chlorophyll radical. The electron deficit in the molecule may then be replenished by an electron donor, most commonly water. It is this passing of electrons that drives the process of photosynthesis and ultimately provides a mechanism for ATP and NAD(P)H synthesis within the chloroplast (Buchanan, et al, 2000; Heldt, 1997).

When electron carriers are highly reduced and cannot accept excited electrons from chlorophyll, alternative mechanisms for chlorophyll to return to its ground state must be in place to avoid damage (**Fig. 2**). The release of excitation energy in the form of light is called fluorescence. Commonly, chlorophyll fluorescence is used as an accurate measure of electron transport efficiency and PSII redox status. The changes in chlorophyll *a* fluorescence are described using a fluorescence induction curve (Hall and Rao, 1994).

The fluorescence induction curve is divided into several components, each which describes a particular aspect of reaction center photochemistry. The term, F_o, represents the fluorescence of all open reaction centers following dark

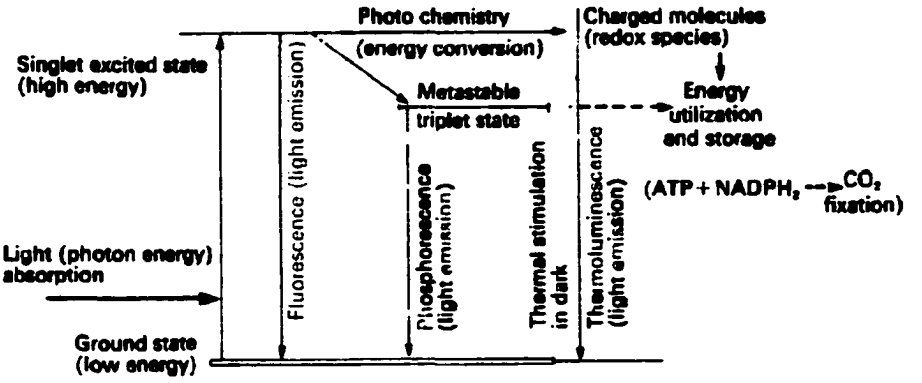

Fig. 2. Schematic diagram of light absorption and energy dissipation by photosynthetic components (from Hall and Rao, 1994).

acclimation. The variable fluorescence (F_v) is the difference in fluorescence level between the maximum fluorescence (F_m; all electron carriers of the cytochrome b_6f complex are reduced/reaction centers are closed) and F_o, and is commonly referred to as photochemical fluorescence quenching. Hall and Rao (1994) assert that the photochemical yield of PSII is equal to the ratio between F_v and F_m (Fv/Fm). Under stress conditions, electron transport molecules may become highly reduced and damage may occur as a result of exposure to excess energy and the formation of reactive molecules. Specific damage to PSII electron transport efficiency is indicated by decreases in Fv/Fm values. In nature, decreases in Fv/Fm may also be indicative of regulatory changes in nonphotochemical quenching during acclimation processes (Niyogi, 1999).

In addition to electron transfer and fluorescence, chlorophyll may return to its ground state through dissipation of energy as heat (via changes in vibration and rotation energy), phosphorescence (requiring a spin reversal from the triplet state), or the excitation of a chromatophore within the photosynthetic apparatus (such as the

pigment antenna system). Chromatophores within the photosynthetic apparatus are more commonly referred to as the pigment antenna system and play an important role in photosynthetic efficiency.

The antenna pigments act as an energy funnel within the light harvesting complexes, comprised of chromaphores which pass solar energy through sympathetic vibration (i.e. Förster mechanism) to the chlorophyll *a* reaction center (Buchanan, et al., 2000). Photosynthetic efficiency is increased by the antenna system as the various pigments absorb overlapping wavelengths of light (making greater use of total available energy). Carotenoids are important functional and structural components of the light harvesting antenna system (Niyogi, 1999; Frank and Codgell, 1996; Young and Britton, 1992). The light harvesting complexes of the reactions centers of PSI and PSII are rich in β-carotene, while the peripheral light harvesting complexes contain a high number of xanthophylls (oxygenated carotenoids; Niyogi, 1999; Young and Britton, 1992).

The role of β-carotene in photosynthesis is most commonly described in terms of the thylakoid-associated light harvesting complexes. As a part of the light reactions, β-carotene is one of several accessory pigments responsible for

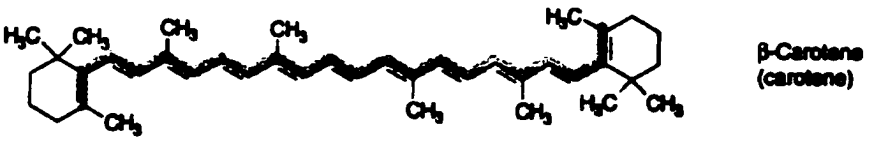

Fig. 3. Molecular structure of β-carotene (Heldt, 1997).

the transfer of excitation energy to chlorophyll. Carotenoids play a variety of roles in addition to light capture in photosynthesis. Frank and Cogdell (1996) have suggested that there are five primary roles of carotenoids in photosynthesis: 1) light harvesting through singlet state energy transfer, 2) photoprotection through the quenching of chlorophyll triplet states, 3) singlet oxygen scavenging, 4) excess energy dissipation (lost as heat), and 5) structure stabilization for the light harvesting complexes.

Carotenoids exhibit high absorbance of visible light at 675nm (when attached to a protein) and from 487-436nm, with some wavelengths continuing to be absorbed in the blue to ultraviolet-a (UV-A; 324-400nm) portion of the spectrum (Siefermann-Harms, 1987). The filtration of blue to UV-A wavelengths may prove to be another important protective function of carotenoids in photosynthesis. The efficiency of energy transfer and utilization in photosynthesis may be described using photosynthesis-irradiance (PI) response curves and monitoring net photosynthesis as total oxygen evolution. In addition, responses to environmental stresses may be observed as changes in light limited to light saturated photosynthetic rates.

Geider and Osborne (1992) have discussed that the initial slope of a PI curve is often determined by light harvesting, while the maximum rate of photosynthesis (P_{max}) is determined by dark cycle reactions (i.e. enzyme activity during carbon fixation and the regeneration of substrates). Theoretically, if light- limited to light-saturated photosynthesis is presented as a ratio under stress conditions, increases in the ratio would be indicative of damage to RubisCO and decreases in the ratio would be indicative of damage to electron transport (resulting in lower electron transfer

efficiency). Changes in this ratio would also potentially indicate acclimation processes to particular environmental conditions, as well.

Ultraviolet irradiance and potential metabolic damage

The ultraviolet spectrum, with extremely high wavelength frequency, has traditionally been divided into UV-A (320-400nm), UV-B (280-320nm) and UV-C (190-280nm). The vast majority of all UV-C wavelengths in solar radiation are absorbed within the atmosphere, hence these wavelengths are not of biological concern to natural systems. Ultraviolet-A photons possess the least energy of the UV spectrum, however, approximately 95% of near UV solar irradiance reaching the earth's surface is UV-A (Holm-Hansen, 1997). Ultraviolet-B comprises approximately 5% of near UV as the majority of UV-B wavelengths are absorbed within the ozone layer of the atmosphere (Smith and Cullen, 1995).

As a result of advances in atmospheric monitoring technology, it has been established that there is reason for concern with regard to ozone depletion. The use of organic halide-containing compounds in consumer products and the subsequent release of these compounds into the troposphere has created a seasonal Antarctic ozone "hole", allowing a much greater irradience of UV-B irradiance on the earth's surface in affected regions of the globe (Franklin and Forster, 1997; Booth, et al., 1997). Although there has been an increase in the attention given to the effects of UV-B on metabolism, UV-A effects have remained largely unexplored even though there is evidence that UV-A may play an important role in plant metabolic functions.

Extremely high energy UV-B irradiance has been correlated with damage to a wide array of metabolic targets including nucleic acids, proteins, membrane lipids, cytoskeleton elements and photosystem II (PSII). In higher plants, Van Hasselt, et al. (1996) found that pea leaves exposed to UV-B exhibited a decrease in Fv/Fm and an increase in the relaxation half-time ($t_{1/2}$) of chlorophyll. The "relaxation half-time" is characterized as the relaxation of the electric field across the thylakoid membrane with a $t_{1/2}$ dependent on the permeability of ions. Changes in $t_{1/2}$ due to UV-B exposure may be related to Q-cycle damage (i.e. damage to electron transport capacity within the cytochrome b_6f complex), decreased PSII reaction centers (from UV-B damage to the D1 protein), or increases in thylakoid proton permeability (by direct damage to thylakoid proteins; Van Hasselt, et al., 1996). In addition, UV-B induced lipid peroxidation as a secondary effect of photosynthetic damage and radical formation. Radical formation was observed, in part, as an increase in electrolyte leakage, which was amplified in dark conditions (resulting from damage to the plasma membrane; Van Hasselt, et al., 1996).

In simpler photoautotrophs, such as microscopic algae, effects from UV-B exposure have been observed, as well. Lesser (1996a,b) observed the effect of both elevated temperature and UV exposure in symbiotic dinoflagellates and found that growth rates and chlorophyll *a* concentrations decreased as a result of UV exposure, while the carbon:nitrogen ratio increased. In addition, concentrations of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) increased with UV exposure as did the activity of both superoxide dismutase (SOD) and ascorbate peroxidase (APX). Although there did not appear to be an effect on the quantum yield of photosynthesis from UV alone,

there was a decrease in Fv/Fm when temperature was increased. Lesser (1996a,b) observed decreases in RubisCO activity and maximum photosynthesis (P_{max}). This damage was minimized upon the addition of the antioxidants, ascorbate and catalase, an indication of the involvement of ROS.

Malanga and Puntarulo (1995) looked at the effects of UV-B on oxidative stress and antioxidant content in the green alga, *Chlorella vulgaris* and found dramatic increases in oxygen radical generation (284%) and lipid peroxidation (145%) following UV-B exposure. Dramatic increases were also observed in SOD and catalase activities (40% and 500%, respectively), as well as, the antioxidants α-tocopherol and β-carotene. Although the photoreducing capacity of chloroplasts appeared to decrease following UV-B exposure, the was an increase in both the size and starch accumulation of cells. The researchers suggest that oxidative stress conditions related to low irradience UV-B exposure elicit a protective response through increased antioxidant activity.

In 1997, Malanga examined the effect of UV-B specifically on chloroplast processes from *C. vulgaris* related to oxidative damage. This research found that doses of high irradience UV-B significantly increased radical production within the chloroplast membranes, however, SOD activity decreased following exposure and there was no observable change in APX activity or ascorbic acid levels. Also, β-carotene concentrations were unaffected by UV-B, while α-tocopherol concentrations increased four-fold. This research suggests that the oxidative damage from UV-B compromises chloroplast function and integrity and the antioxidant response signals an increase in the concentration of α-tocopherol.

The research on UV-A, although limited, implies that these wavelengths play an important role in plant metabolic functions. For example, Helbling, et al. (1996) found that UV-A was responsible for greater than 50% of UV induced photosynthetic inhibition in oceanic plankton populations. Jeffrey and Mitchell (1997) found that both UV-A and UV-B inhibit both primary and heterotrophic production in marine waters, and UV radiation may interact with UV-absorbing compounds (eg. mycosporine-like amino acids) and endogenous photosensitizers that can modulate the effect on other macromolecules. Relative to UV-B, UV-A comprises a much greater portion of surface-level UV and is responsible for a significant portion of the pathogenic effect of sunlight. Although UV-A and UV-B both inhibit growth and productivity, most effects are attributed to DNA damage (UV-B directly, UV-A indirectly).

Hermann, et al. (1997) compared photosynthetic inhibition by solar radiation in *Dunaliella salina* relative to UV-A, UV-B and PAR wavelengths and found that sensitivity to UV exposure was variable. The research showed that, on a quantum basis, UV-B is a more effective inhibitor of photosynthesis than UV-A. This assessment makes sense from a wavelength frequency perspective; however, it does not accurately reflect natural conditions. In a natural sunlight environment, at sea level there are proportionally many more UV-A photons than UV-B based on the absorption of most UV-B wavelengths by atmospheric ozone. Hence, the effect of natural exposure on physiology must be compared based on the total energetics (and quantity of incident light) using weighted doses from biological weighing functions of individual wavelengths.

Goyal and Tolbert (1991) made two interesting observations with respect to UV inhibition of the dissolved inorganic carbon (DIC) concentrating mechanism(s) and alternative respiration in unicellular green algae. First, the research indicated that the induction and/or development of the DIC pump (specifically, via ATPase inactivation) is inhibited by low levels of UV-B, but not UV-A (with maximum inhibition around 290nm). Second, UV-A, but not UV-B, inhibited the capacity of alternative respiration in both algae and leaves of higher plants. As both DIC concentrating mechanisms and alternative respiration are inhibited by SHAM, it was suggested that these two processes could be related (Goyal and Tolbert, 1991).

Reactive oxygen formation in cells (specifically, H_2O_2)

Halliwell and Gutteridge (1999) define a free radical as being any molecular species capable of independent existence that contains one or more unpaired electrons in the atomic orbital. Radicals may be formed through the loss or gain of a single electron by a non-radical molecule. When covalent bonds are broken during metabolic processes, one of two types of bond fission may occur: homolytic or heterolytic. During homolytic bond fission, a covalent bond is broken and one electron from each shared pair remains with each atom resulting in the formation of two radical species. In heterolytic bond fission, one atom receives both electrons when a covalent bond is broken, thus, no radical species are formed. Jahnke and Frenkel (1975) established that the relationship between homolytic bond fission and

superoxide production is mediated by saponified chlorophyll in higher plants (spinach and bean).

Reactive oxygen molecules are formed during the conversion of oxygen (O_2) to water (H_2O), as well as, the relatively stable oxidant, hydrogen peroxide (H_2O_2; Scandalios, et al., 1997). Once formed, ROI's have the potential to cause morphological, physiological, biochemical and molecular damage within the cell (Asada, 1999; Malanga, et al., 1997; Allen, 1995; Foyer and Mullineaux, 1994; Asada and Takahashi, 1987). There are two types of reactions that generate H_2O_2 in peroxisomes, mitochondria, and chloroplasts: 1) the univalent reducation of oxygen to form superoxide radicals followed by a dismutation into H_2O_2 and O_2 and, 2) divalent reduction of molecular O_2 enzymatically (i.e. glucose oxidase Ishikawa, et al., 1993). Since H_2O_2 can freely diffuse across cell membranes there must be a coordinated effort by several enzymes to scavenge it completely to prevent metabolic damage from metal-catalyzed Fenton reactions (formation of hydoxyl radicals via the oxidation of iron or copper; Asada, 1999; Collen, et al., 1995).

Formation of H_2O_2 in the cell occurs primarily in peroxisomes, chloroplasts, and mitochondria, although limited quantities have been detected in the cytosol, the endoplasmic reticulum, and the nucleus (Scandalios, 1994). Chloroplasts are a major contributor to the production of H_2O_2, as approximately 10-20% of the electrons passing through photosystem I reduce O_2 to superoxide. Superoxide is then rapidly reduced to H_2O_2 by superoxide dismutase (SOD) within the chloroplast (Asada and Takahashi, 1987). The light-driven reduction of O_2 to superoxide in the chloroplast is known as the Mehler reaction.

Peroxisomes contain oxidases involved in a variety of metabolic reactions within the cell and, as a consequence, produce much of the H_2O_2 found in cells (**Fig. 4**). In the process of removing two electrons from substrates, oxidases ultimately

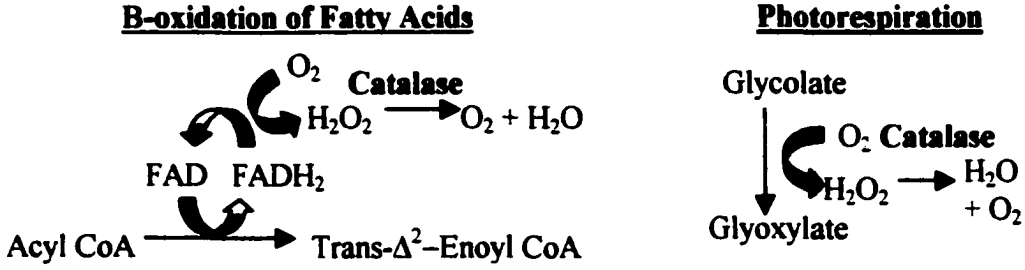

Fig. 4. Summary of the hydrogen peroxide producing reactions contained within the microbodies of photosynthetic organisms.

transfer the electrons to O_2, forming H_2O_2 (Scandalios, 1994). During photorespiration, hydrogen peroxide is generated through the divalent reduction of dioxygen by glycolate oxidase. Production of H_2O_2 in peroxisomes is directly proportional to the rate of phosphoglycolate production during the oxygenase reaction of RubisCO. Phosphoglycolate is converted to glycolate by a specific phosphatase in chloroplasts and is then transported to the peroxisome where glycolate is oxidized to glyoxylate, producing H_2O_2 (Buchanan, et al., 2000). As a result, photorespiratory H_2O_2 production in peroxisomes is variable depending on the environmental and physiological conditions that effect photorespiration (Asada and Takahashi, 1987). Some species of higher plants have evolved carbon concentrating mechanisms, such as C4 or crassulacean acid metabolism, to minimize the oxygenase activity of RubisCO and thus, photorespiration. Most cyanobacteria and algae have carbon concentrating mechanisms as well, consisting of an extremely effective enzyme/pump

system to minimize photorespiration in cells (Heldt, 1997; Sultemeyer, et al., 1993; Goyal and Tolbert, 1991; Dionisio, et al., 1989).

In addition to photorespiration, H_2O_2 is also produced within peroxisomes during the β-oxidation of fatty acids (**Fig. 4**). In the process of fatty acid β-oxidation, $FADH_2$ is produced during the conversion of Acyl CoA to *trans*-Δ^2-enoyl CoA. In the regeneration of FAD, $FADH_2$ interacts with oxygen producing H_2O_2. In order to protect the cell from damage related to the production of H_2O_2 peroxisomes contain the scavenging enzyme, catalase (Scandalios, 1994).

In mitochondria, $O_2^{\cdot-}$ is believed to be the precursor to H_2O_2 as a result of electron "leaks" from the electron transport chain of aerobic respiration. As the flow of electrons increases, the formation of H_2O_2 increases as a result of the increasing production of $O_2^{\cdot-}$. Much as in chloroplasts, H_2O_2 production is associated with the action of SOD in response to $O_2^{\cdot-}$ (Ishikawa, et al., 1993; Scandalios, 1994). Ishikawa, et al. (1993) suggest that the generation of $O_2^{\cdot-}$ in mitochondria occurs in the regions of NADH dehydrogenase and ubiquinone-cytochrome b segments of respiration. Intact mitochondria from *Euglena gracilis* were observed to determine the origin of H_2O_2 related to respiratory electron flux. When respiratory substrates such as malate, succinate, lactate, glutamate, and glycolate were omitted from the reaction mixture in the control, H_2O_2 generation was not observed indicating that the production of H_2O_2 is directly related to flux through the respiratory chain and availability of respiratory substrates (Ishikawa, et al., 1993).

In normal chloroplasts there is no divalent reduction of dioxygen to produce H_2O_2 (Collen, et al., 1995). The are several mechanisms by which H_2O_2 is generated in chloroplasts. The majority of H_2O_2 appears to be derived from superoxide and the activity of superoxide dismutase (**Fig. 5**; SOD). The thylakoid membrane-associated reactions are responsible for the generation of H_2O_2 within photosystem I (PSI) on the stromal surface of the membrane. Superoxide formation is generally considered to arise in PSI, as oxygen is reduced primarily by PSI components such as ferredoxin and ferredoxin/$NADP^+$ oxido-reductase (Foyer, et al., 1997). A limited quantity of superoxide is formed from PSII redox processes, as well. The reactions by which H_2O_2 is produced from $O_2^{\cdot-}$ using SOD are commonly referred to as the Mehler reaction.

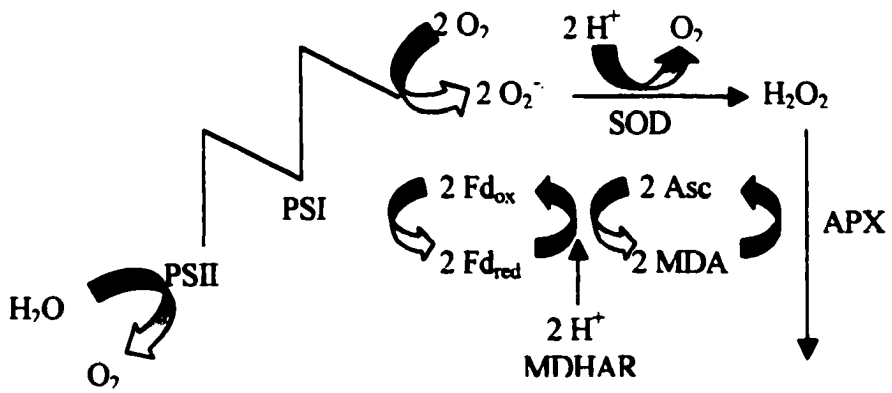

Fig. 5. Summary of the hydrogen peroxide producing reactions contained within the chloroplasts of photosynthetic organisms. SOD, superoxide dismutase; APX , ascorbate peroxidase; MDA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; Asc, ascorbate.

In addition to formation of H_2O_2 by the reduction of $O_2^{\cdot-}$ via SOD, some H_2O_2 in chloroplasts is generated through the reaction: $HO_2 + NAD(P)H \rightarrow H_2O_2 + NAD(P)^{\cdot}$. The NAD radical then univalently reduces dioxygen, producing $O_2^{\cdot-}$ once again. This

series of reactions occurs in limited quantities only when the pH is low enough that HO_2 is the predominant form of oxygen in the chloroplast, such as the aprotic environment found in membranes (Asada and Takahashi, 1987).

Other means of H_2O_2 production have been identified in algae and cyanobacteria, in addition to the traditional chloroplast reactions of higher plants. In contrast to the SOD of higher plants, *Euglena* sp. contain Fe- and Mn-superoxide dismutases. The SOD enzymes within *Euglena* sp. are predominantly localized within the chloroplast, where even low concentrations of H_2O_2 can cause irreversible damage to photosynthesis. Outside of the chloroplast, in the cytosol, H_2O_2 damage may be repaired by the cell, therefore, there is not a need for the elaborate scavenging system found in the chloroplast (Ishikawa, et al., 1993).

The formation of H_2O_2 within plant cells is a potentially toxic process from either direct (i.e. enzyme inactivation) or indirect damage (i.e. the production of highly reactive free radicals during H_2O_2 decomposition). Perhaps the most significant direct damage caused by H_2O_2 is the inhibition of photosynthesis at relatively low concentrations. In spinach, researchers have demonstrated that the addition of 10μM H_2O_2 decreased photoassimilation of $^{14}CO_2$ to 50% when scavenging enzymes of H_2O_2 were suppressed using cyanide (Kaiser, 1976). H_2O_2 disrupts the photosynthetic process by inhibiting a wide array of enzymes in the Calvin cycle, such as fructose bisphosphatase (Halliwell and Gutteridge, 1999), ribulose phosphate kinase (Collen and Pedersen, 1996), and ribulose bisphosphate carboxylase/oxygenase (Collen and Pedersen, 1996). Collen and Pedersen (1996) have suggested that superoxide dismutases may also be sensitive to the accumulation of H_2O_2 in cells. It appears that

H_2O_2 inactivates these enzymes by oxidation of essential thiol (-SH) groups (Halliwell and Gutteridge, 1999).

Although significant damage to photosynthesis has been demonstrated in higher plants, Takeda, et al. (1995) have suggested that algae actually appear to be resistant to this form of damage from H_2O_2. Takeda, et al. (1995) demonstrate the effects of 1mM and 0.1mM H_2O_2 on CO_2 fixation and thiol-modulated enzyme inhibition, respectively, in *Euglena* and *Chlamydomonas*. While 10 μM H_2O_2 resulted in a 50% inhibition of CO_2 fixation in spinach chloroplasts, 1mM H_2O_2 only reduced CO_2 fixation in *Euglena* and *Chlamydomonas* by 30% and 45% respectively, and in cyanobacteria treated with 1mM hydroxylamine, the fixation of CO_2 was only inhibited 25-40%. Also, in contrast to the inhibition of thiol-modulated enzymes (i.e. fructose bisphosphatase, NADP-glyceraldehyde-3-phosphate dehydrogenase, and ribulose-5-phosphate kinase) in spinach chloroplasts by low concentrations of H_2O_2, algal and cyanobacterial cells exhibited resistance to damage and a much greater tolerance to H_2O_2 accumulation. Takeda, et al. (1995) suggest that the difference in H_2O_2 susceptibility may be related to structural differences in the vicinity of the thiol groups involved in light-dependent activation between enzymes of algal and higher plant chloroplasts. The researchers also suggest the possibility that the algal enzymes lack the Cys residues that are involved in the light-dependent regulation of enzymes in higher plants, however, additional testing must be performed to establish which of these theories is correct.

Hydrogen peroxide is a relatively stable oxidant and not highly reactive, however, the transition-metal-catalyzed Fenton reaction can form a highly reactive hydroxyl

radical ($^{\cdot}OH$) through the reaction: $Fe^{2+}+H_2O_2$ ----> $Fe^{3+}+{}^{\cdot}OH + OH^{-}$ (Asada and Takahashi, 1987; Halliwell and Gutteridge, 1999). The Fenton reaction should not be confused with the non-metal or metal catalyzed Haber-Weiss reaction which depends on the conversion of $O_2^{\cdot-}$ to form H_2O_2 and subsequently, $^{\cdot}OH$ (Allen, 1995). Typically, chelation of transition metals prevents the Fenton reaction from occurring. However, when transition metals are not bound within the cell, the reaction of reduced iron (or copper) and hydrogen peroxide results in the oxidation of the metal and the formation of a hydroxyl radical.

Hydroxyl radicals may also be formed from H_2O_2 exposure to ultraviolet radiation and subsequent homolytic fission (resulting in the formation of $2\,^{\cdot}OH$). The hydroxyl radical can then cause damage to proteins, fatty acids and bilayer lipid membranes resulting in an array of physiological damage within the cell (Scandalios, et al., 1997). In addition, UV related formation of $OH^{\cdot}$ from H_2O_2 appears to effect DNA, most frequently causing single strand breaks and DNA protein cross links (Halliwell and Gutteridge, 1999).

Reactive oxygen scavenging in cells

In general, depending on the site of formation, H_2O_2 may be scavenged in several ways to prevent direct or indirect damage from the formation of hydroxyl radicals: 1) peroxidases scavenge H_2O_2 through the oxidation of ascorbate or glutathione, which is then regenerated enzymatically by reduction using NADPH or NADH or, 2)

catalase scavenges H_2O_2 and dismutes (disproportionates) the molecule into O_2 and H_2O (Takeda, et al., 1997; Collen and Pedersen, 1996).

Halliwell and Gutteridge (1999) report that there are five main problems associated with the production of H_2O_2 in chloroplasts requiring the intervention of antioxidant scavenging enzymes. First, the internal oxygen concentration in the light is always greater than the surrounding atmosphere from oxygen production in PSII (thus resulting in an increased opportunity for reactive oxygen species to be formed). Second, the lipids found in the chloroplast envelope and thylakoid membranes contain a high number of polyunsaturated fatty acids (i.e. fatty acids with a large number of double bonds) and thus are susceptible to peroxidation from reactive oxygen species. Third, illuminated chloroplasts can sensitize the formation of singlet oxygen, which is especially damaging to chloroplast lipids. Fourth, the electron transport chain of photosynthesis can leak electrons into oxygen, and thus cause the Mehler reaction to occur forming dangerous hydroxyl radicals. Finally, oxygen can cause photorespiration by affecting the abilities of the enzyme Rubisco (by favoring the oxygenase function of the enzyme). In order to prevent damage to the chloroplast due to oxygen interactions, plants have evolved a series of enzymatic scavenging systems.

Ascorbate peroxidase (APX) has traditionally been identified as the primary peroxidase responsible for the detoxification of hydrogen peroxide within plant chloroplasts and cytoplasm (Mehlhorn, et al., 1996). APX is a heme-containing protein which is substrate specific for ascorbate as an electron donor (Foyer, 1994). APX specifically catalyses the reaction $2H^+$+ascorbate+H_2O_2 ----> 2 mono-

dehydroascorbate +2H_2O. Mono-dehydroascorbate is then rapidly reduced by NADH in an enzymatic reaction (Miyake, et al., 1991). Plants also have peroxidases which are able to use other electron donors. As previously described, $O_2^{\cdot-}$ is dismuted by chloroplast-associated SOD and the resulting H_2O_2 is scavenged by thylakoid-bound APX. Reactive oxygen species that escape destruction by thylakoid-bound APX are then scavenging by stromal SOD and APX. Monodehydroascorbate radicals (MDA) produced by APX are converted to ascorbic acid through reactions with ferredoxin or monodehydroascorbate reductase (MDHAR). Reduction of dehydroascorbate to ascorbic acid is catalyzed by dehydroascorbate reductase through the ascorbate:glutathione pathway (Noctor and Foyer, 1998).

Cytosolic and chloroplastic forms of APX differ in substrate specificity, pH optima, and sensitivity to ascorbate depletion (Allen, 1995). Past research has found that cytosolic APXs are encoded by nuclear genes and a number of cDNAs have been isolated and characterized from plants such as pea and *Arabidopsis* . In contrast, chloroplast enzymes may be encoded either in the nucleus or within the plastid genome. Within the chloroplast, there are two distinct forms of APX, stromal and thalakoid-bound. Stromal and thylakoid APX are found as monomers, but several cytosolic APXs occur as homodimers (Foyer, 1997). Although cDNAs from cytosolic APXs have been isolated, no cDNAs that encode chloroplastic APXs have yet been reported. Partial amino acid sequences have been determined from species such as tea (stromal APX) and spinach (thylakoid-bound APX). Sequences reveal that the amino acids in the amino terminal region of thylakoid APX (spinach) and the stromal APX (tea) show a high degree of homology to each other, but differ from those of

cytosolic APX. In addition, cytosolic APX and stromal APX share a homologous sequence around the proximal and distal histidine residues (Asada, et al., 1993).

Miyake and Asada (1992) and Miyake, Cao, and Asada (1993) purified APX from spinach chloroplasts and have characterized thylakoid-bound APX. Miyake and Asada (1992) characterized APX and the associated MDA reactions, as well as, summarizing the microlocalization of H_2O_2 scavenging system. Research suggests that thylakoid bound APX is the primary scavenger of H_2O_2 photoproduced from the thylakoids. This is based on the inhibition of APX with KCN, resulting in an absence of photoreduction of thylakoid H_2O_2. In addition, Miyake and Asada (1992) determined that ascorbate is photoregenerated in the thylakoids from the MDA radicals produced in a reaction of APX for the scavenging of H_2O_2.

Miyake, Cao, and Asada (1993) developed this research one step further by identifying the molecular properties of thylakoid-bound APX. This research found that thylakoid-bound APX from spinach shared similar molecular and enzymatic properties to those of stromal APXs found in spinach and tea. One of the properties these two forms of the enzyme share is rapid inactivation in an ascorbate depleted medium. Thylakoid-bound APX was also determined to have a higher molecular weight than the stromal form. Research shows that there is an amino-terminal sequence homology between thylakoid-bound APX and stromal APX, thus suggesting that this region does not seem to be a domain required for the binding to thylakoid membranes. Although sequence homology and similar molecular properties were found between chloroplastic APXs, significant differences were found between these and cytosolic APX. Researchers have theorized that the ancestor of APX first

diverged to give cytosolic and chloroplastic APX and then the chloroplastic APX then diverged later to produce the thylakoid-bound and stromal forms of APX (Miyake, et al., 1991).

The regeneration of ascorbate is an important requirement for the continued antioxidative function of the ascorbate pool in cells. As previously mentioned, within chloroplasts MDA radicals produced by APX are converted to ascorbic acid through reactions with ferredoxin or MDHAR. This is the first step in the regeneration of ascorbate in chloroplasts. MDA radicals that escape reduction disproportionate spontaneously to produce ascorbate and dehydroascorbate (DHA). An NAD(P)H dependent MDAR is found in the stroma, however, in which DHA is recycled to ascorbate using reduced glutathione (GSH) in a reaction catalyzed by DHAR. Oxidized glutathione (GSSG) is then reduced by glutathione reductase using NADPH generated by the electron transport chain. The intracellular distribution of ascorbate and GSH is related to the sites within the cell where these antioxidants are synthesized, as well as, the mechanisms of translocation from these sites of synthesis to where they are needed. In typical plants with C3 metabolism, ascorbate concentrations were found to be the highest in protoplasts and chloroplasts, and lowest in thylakoid membranes (Foyer, et al., 1997).

In mitochondria, H_2O_2 is produced primarily through the dismutation of superoxide (via SOD). During normal mitochondrial respiration, superoxide is produced when the redox cycling of ubiquinone-complex III is restricted and components of the electron transport chain autoxidize in the presence of molecular

oxygen forming superoxide (Purvis, 1997). Once scavenged by SOD, superoxide is then converted to H_2O_2 which is scavenged by catalase.

The alternative oxidase pathway found in plant mitochondria allows the transfer of electrons from ubiquinol to oxygen that bypasses cytochrome *c* oxidase. Although insensitive to classic cytochrome *c* oxidase and complex III inhibitors, the alternative oxidase is selectively inhibited by salicylhydroxamic acid (SHAM; Buchanan et al., 2000). No proton electrochemical gradient is formed when electrons are passed by the alternative oxidase to oxygen (generating water). Hence, all free energy released during electron flow is lost as heat and may not be used for ATP synthesis. One function of the alternative oxidase pathway in mitochondria may be to limit the production of superoxide in stress conditions (Purvis, 1997).

The main site of H_2O_2 production within the cell is the microbody, or more specifically, the peroxisome. As previously described, several processes (photorespiration and the β-oxidation of fatty acids) occur within microbodies that result in the formation of H_2O_2, and catalase is the enzyme responsible for scavenging and protection. The enzyme catalase is unique in many respects and will be discussed in much greater detail.

Characteristics of the enzyme, catalase

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6; CAT) is a tetrameric, heme-containing enzyme, capable of acting in two ways. First, at low concentrations, catalase may act peroxidatively to oxidize a hydrogen donor, and second, at high concentrations, catalase rapidly degrades H_2O_2 acting as both a hydrogen donor and

acceptor (Scandalios, 1994; Aebi, 1984). Catalase exhibits a high K_m for H_2O_2 as a substrate, however, though is it not possible to saturate the enzyme with reasonable substrate concentrations (up to 5M H_2O_2), concentrations above 0.1M H_2O_2 have been shown to cause the rapid inactivation of catalase (Aebi, 1984). Aebi (1984) attributes this inactivation of catalase to the conversion of the active enzyme-H_2O_2 complex I to inactive complexes II and III. Aebi (1984) describes the enzymatic decomposition of H_2O_2 as "a first-order reaction, the rate of which is always proportional to the peroxide concentration present."

Catalase synthesis may be sensitive to light, with prolonged exposure resulting in photoinactivation. Cheng, et al. (1981) found that light corresponding to the maximal absorbance of the heme site of catalase (405nm) was the most effective in the inactivation of the enzyme (**Fig. 6**). Cheng, et al. (1981) also concluded that catalase was unable to scavenge reactive oxygen molecules after photoinhibition due

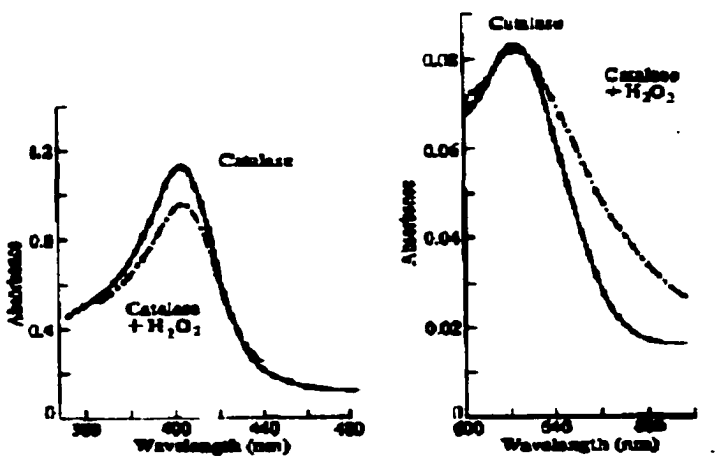

Fig. 6. Absorbance spectra of the heme enzyme, catalase, isolated from rat liver (A) and purified compound I (B). The absorbance peak at approximately 400 nm is referred to as the Soret band, a feature of most heme proteins (from Halliwell and Gutteridge, 1999).

to the redox transformation of catalase. This research demonstrated that the conversion from ferricatalase to compound I occurs during photoinhibition changing the active site of the enzyme and thus, making catalase ineffective at ROS scavenging.

Feierabend and Engle (1986) examined photoinhibition of catalase, *in vivo*, to compare to the earlier work of Cheng, et al. (1981), specifically, the effect of prolonged light exposure on catalase activity in rye leaf chloroplasts and peroxisomes. Feierabend and Engle (1986) found that plant catalase was as light sensitive as the liver catalase used by Cheng, et al. This research also suggests that photoinactivation of catalase may be an early indication of damage in leaves as well as, enhancing the progress (through the loss of scavenging abilities). Though natural levels of light irradience did not cause photoinactivation of catalase in these experiments, amplified light irradience and red light did. As a result, Feierabend and Engle (1986) suggest that the inactivation of catalase is mediated through photooxidative processes associated with the heme moieties of the enzyme. Photooxidative processes in the chloroplast and subsequent formation of reactive oxygen may also contribute to the inactivation of catalase, particularly following periods of exposure to increased irradiance (Feierabend and Engel, 1986).

Research has also suggested that isozymes of catalase exhibit different degrees of photoinactivation. Grotjohann, et al. (1997) isolated catalase isozymes CAT2 through CAT8 and found that forms CAT2 - CAT5 were less photosensitive than other forms of the enzyme. Polidoros and Scandalios (1997) examined the difference between the light independent expression of CAT1 genes and light dependent expression of CAT

2 and 3 genes in Maize and found that all three genes did, in fact, exhibit different reactions to light and circadian patterns.

In addition to inactivation due to prolonged light exposure, catalase is sensitive to a variety of environmental stresses including O_2:CO_2 ratio, pH, temperature, ultraviolet light, and salt. Butow, et al. (1994) examined the response of catalase to changes in CO_2:O_2, as well as pH. This research described the effect of three different atmospheres on catalase activity in the freshwater dinoflagellate *Perdinium gatunense*. The data show a marked decrease in catalase activity under conditions of low O_2:CO_2 and an increase in activity under conditions of elevated O_2:CO_2 (even greater than the activity in normal air concentrations). This is particularly relevant to limnological experiments, where O_2:CO_2 ratios may vary greatly during the course of a year.

Butow, et al. (1994) also examined the effect of pH on the activity of catalase, both *in vivo* and *in vitro*. This research suggests that catalase exhibits a fairly broad effective pH range, approximately pH 6 - 12. Although this is a fairly broad peak, it should be noted that the range is primarily neutral-alkaline, and acidic pH (below approximately 6.5) results in a sharp decline in catalase activity.

Volk and Feierabend (1989) examined the effect of low temperature on photoinactivation of catalase in rye leaves and found that although catalase was inactivated at low temperatures, recovery time was quick once the temperature was increased. It should be noted that although catalase activity drops dramatically during prolonged incubation at 4°C, there is an immediate increase in activity under light and dark conditions once the temperature is increased. This is an indication that the

inhibition of catalase by cold temperature acts independent of the effect of light exposure.

As previously described, Malanga and Puntarulo (1995) observed the effects of ultraviolet-B (UVB) radiation on antioxidants in *Chlorella vulgaris*. Both catalase and SOD showed a marked increase in activity following exposure to UVB. It may be asserted that this increase in activity is related to the ability of UVB to cause the formation of reactive molecules within the cell. As the number of ROI's increases due to light exposure, the production of scavengers also increases proportionally to prevent cellular damage.

Finally, Streb and Feierabend (1995) examined the effect of NaCl on photoinactivation of catalase in rye leaves, specifically, the changes in the contents of various cell components (including total peroxide, H_2O_2, catalase, and glutathione). The compound used in the second set of experiments, cycloheximide, is a translation inhibitor which closely mimicked the symptoms of photooxidative damage seen in NaCl treated leaves. The findings of this research imply that NaCl induced damage in light was a result of inhibition of protein synthesis. When inhibition of protein synthesis occurs, there is no regeneration of catalase, and thus, activity decreases rapidly.

Catalase has been found in all eukaryotic cells, and research indicates that the primary role of this enzyme is the degradation of H_2O_2 and reactive oxygen intermediates, thus preventing associated damage from the formation of hydroxyl radicals. Catalase does exhibit sensitivity to light and a variety of environmental

factors, however, the quick rate of turnover and ability to recover from damage readily make catalase one of the most efficient scavenging systems within the cell.

Applications for Dunaliella sp. in physiological testing

The species *Dunaliella* is well suited to physiological testing for several reasons. First, the absence of a cell wall facilitates physical manipulation for biochemical analysis. Second, *Dunaliella* is extremely halotolerant, with research suggesting it may be the most halotolerant eukaryotic microorganisms known (Brock, 1975). Finally, two species of *Dunaliella*, *D. salina* and *D. bardawil*, have been shown to accumulate copious amounts of inter-thylakoid β-carotene in response to stress conditions.

Brown and Borowitzka (1979) summarize the physiological range of tolerance for many species of *Dunaliella* and a variety of environmental conditions, including salinity, pH, temperature, and light. Through acclimation (and most likely antioxidant changes), *D. tertiolecta* was found capable of existence in a 0.05-3.0 M salinity range (Jahnke and White, unpublished). The species, *D. salina*, *D. viridis*, and *D. parva* are commonly found in highly saline lakes (such as the Great Salt Lake) and are described as halophilic (although fundamentally different from halophilic bacteria). *Dunaliella* is unique, as the genus is capable of osmotic adjustment through the synthesis and accumulation of glycerol within the cell. The presence of glycerol eliminates the need for salts as osmoregulatory solutes and may act as an osmoticum without inhibiting cytoplasmic and membrane-bound enzymes at physiological concentrations (Brown and Borowitzka, 1979).

Brown and Borowitzka (1979) describe optimal light and temperature conditions as being species specific and often, related to salt tolerance. For example, the more salt-tolerant species *D. salina* and *D. viridis* grow optimally at higher temperatures (30° and 37°C, respectively) and high light intensities (10,000-35,000 lx), while the marine species *D. tertiolecta* grows best at 20°C. Interestingly, red light is favored for growth by *D. salina* and there is an actual depression in photosynthetic activity at the blue end of the spectrum. This depression in photosynthesis is most pronounced in *D. salina* grown in high salt concentrations than in low concentrations (Brown and Borowitzka, 1979).

It is believed that *D. bardawil* and *D. salina* Teod. accumulate copious amounts of β-carotene to protect against high light damage to photosynthesis in addition to the traditional light harvesting complex β-carotene (Ben-Amotz, et al., 1989). Unlike the thylakoid-associated β-carotene of the light harvesting complex, however, this massive accumulation occurs in the inter-thylakoid spaces of the chloroplast and is found as all *-trans* and 9-*cis*-β-carotene forms (Ben-Amotz, et al., 1989). Ben-Amotz, et al. (1989) found that photo-induced β-carotene accumulation in *D. bardawil* was based on light quantity and not quality. In essence, any wavelength within the visible spectrum can induce β-carotene accumulation as long as the irradience is high enough.

In addition to high irradience PAR, other factors have been observed which elicit a similar response in *D. bardawil*, including high salt concentrations, extreme temperatures, nutrient deficiencies (mainly nitrogen and sulfur), and UV-A irradiance (Jahnke, 1999; Ben-Amotz and Avron, 1983). *Dunaliella bardawil* has been shown to

accumulate β-carotene to at least 8% of its dry weight when grown under these stress conditions.

Ben-Amotz, et al. (1989) have suggested that the mode of action of the accumulated β-carotene in *D. bardawil* is blue light filtration in order to protect photosynthesis from excess irradiation. This research found that the accumulation of β-carotene was effective in preventing photoinhibition. This protection was not associated with the scavenging of ROI's, as the β-carotene globules are not within a reasonable proximity for chlorophyll quenching. Instead, Ben-Amotz found that the mode of action was related to a simple screening effect (of blue light), as no protection occurred when the cells were exposed to high red light conditions.

CHAPTER I

CONTRASTING EFFECTS OF UV-A AND UV-B ON PHOTOSYNTHESIS AND PHOTOPROTECTION OF BETA-CAROTENE IN TWO *DUNALIELLA* SPP.

ABSTRACT

The photosynthetic and antioxidant responses following exposure to either ultraviolet-A or ultraviolet-B were examined and contrasted in two species of the unicellular green alga *Dunaliella.* Species selection was based on the ability of *Dunaliella bardawil* (UTEX 2538) to accumulate large quantities of inter-thylakoid β-carotene during exposure to stress or high irradience photosynthetically active radiation while *Dunaliella salina* (UTEX 200) lacks this ability. Cells were cultured in high and low irradiance environments (150 and 35 μmol photons m^{-2} s^{-1}, respectively) and then exposed to ultraviolet-A (320-400 nm) + PAR or ultraviolet-B (290-320 nm) + PAR, independently, for 24 hour test periods. A potassium chromate solution was found to be an ideal screen for the removal of both ultraviolet-A and ultraviolet-C from ultraviolet-B radiation. Ascorbate concentration and ascorbate peroxidase activity, photosynthetic oxygen evolution and fluorescence parameters were monitored following UV exposure. There was no significant change in photosynthetic or antioxidant parameters following exposure to ultraviolet-B. Ultraviolet-A exposure greatly decreased photosynthetic capacity resulting in a 70% decrease in both Fv/Fm and light limited to light saturated photosynthesis.

Ultraviolet-A also resulted in 50% increases in ascorbate peroxidase activity and ascorbate concentrations. The results suggest that exposure to ultraviolet-A (but not ultraviolet-B) directly affects photosynthesis, observed as a loss of photosystem II electron transport efficiency (and increased radical formation). This research suggests that the accumulated β-carotene in *Dunaliella bardawil* prevents UV-related photosynthetic damage through blue-light/ultraviolet-A absorption and is supported by trends observed for antioxidant enzyme responses.

INTRODUCTION

The recognition of a seasonal hole in the ozone layer in Antarctica (and global decreases in stratospheric ozone) has piqued the interest of researchers with regard to the potential effects of increased ultraviolet-B radiation on the earth's surface. Approximately 95% of near-UV photons are ultraviolet-A (UV-A, 320-400 nm), while the remainder are ultraviolet-B (UV-B 290-320 nm), and both have been shown to elicit physiological responses in a variety of organisms. There has been a great deal of controversy surrounding the effects of specific UV wavelengths on photosynthetic systems with respect to observed responses, as well as the mechanism of damage. Research has demonstrated that exposure to UV-A may result in direct photosynthetic damage. As UV-A exposure is not affected by changes in the ozone layer, only a small fraction of UV research targets these specific responses (Helbling, et al., 1992; Smith and Cullen, 1995; Dring, et al., 1996; Holm-Hansen, 1997). In contrast, UV-B has received a great deal of attention accompanied by variable results on the

biological and physiological effects of exposure. All UV-B lamps produce residual UV-A and ultraviolet-C (UV-C; 240-290 nm) wavelengths. Although cellulose acetate is typically used in order to eliminate UV-C wavelengths, few researchers have adequately addressed the interference of UV-A from these light sources; thus, many UV-B experiments do not use pure UV-B wavelengths (Adamse and Britz, 1992; Middleton and Teramura, 1993; Newsham, et al., 1996). Contrasting reports have been published regarding the effects of UV-B on both photosynthetic processes (Middleton and Teramura, 1993; Nogues and Baker, 1995; van Hasselt, et al., 1996; Figueroa, et al., 1997; Forster, et al., 1997; Hermann, et al., 1997; Allen, et al., 1998; Ghetti, et al., 1998, 1999; Krause, et al., 1999; Heraud and Beardall, 2000; Xiong and Day, 2001) and antioxidant responses (Malanga and Puntarulo, 1995; Lesser, 1996a,b; Malanga, et al., 1997; Mackerness, et al., 1999; Mazza, et al., 1999; Vega and Pizarro, 2000; Estevez, et al., 2001). A new technique has been developed for this research that employs a potassium chromate solution to remove residual UV-A and UV-C thus producing a cleaner UV-B radiation exposure.

Various environmental stresses have been related to the massive accumulation of inter-thylakoid β-carotene in the alga *Dunaliella bardawil* including high irradience light conditions, salinity and temperature extremes, deficiencies in either sulfate or nitrate, and exposure to UV-A wavelengths (Ben-Amotz and Avron, 1983; Jahnke 1999). Jahnke (1999) has shown that *D. bardawil* accumulates massive quantities of β-carotene in response to UV-A exposure (but not UV-B), without a significant reduction in growth rates. Unlike the thylakoid-associated β-carotene, the massive accumulation of β-carotene is inter-thylakoid, in globules that are not capable of

directly quenching the formation of active oxygen molecules (because of the distance from the photosystems). Past research has found that this accumulation of β-carotene may act as a protective mechanism for photosynthesis in high irradience blue and white light conditions, suggesting that β-carotene protects against damage by screening the light-harvesting complexes through absorption of blue/UV-A wavelengths (Ben-Amotz, et al., 1989; Shaish, et al., 1993).

The two plausible mechanisms for photoinhibition are reductive damage to PSII (specifically, Q_A, the primary quinone acceptor of PSII) and oxidative damage (water-splitting) resulting in a stable charge separation (Aro, et al., 1993). Under high-irradiance conditions, PSII membrane-bound protease triggers thylakoid membranes to degrade and release fragments of D_1 protein. Under normal light conditions, *de novo* synthesis of the D_1 protein can balance degradation; hence, there are no observable photoinhibitory effects. In contrast, under high-irradience light conditions active oxygen species are formed (resulting from the rapid flux of electrons from $Pheo^+$ to Q_A, causing the formation of singlet oxygen via P680-Chl triplet state) resulting in the bleaching of P680 and the degradation of the D_1 polypeptide (Aro, et al., 1993). Two β-carotene molecules within the D_1 protein complex have evolved that quench chlorophyll triplets and active oxygen species, thereby reducing the damage to PSII (Hall and Rao, 1994).

In chloroplasts, H_2O_2 is generated by superoxide dismutase (SOD) following superoxide formation from electron transport during the light reactions of photosynthesis (i.e. Mehler reaction; Henry and Hall, 1977; de Jesus, et al., 1989). Although H_2O_2 is a fairly stable oxidant, transition metals that are not bound within

the cell may promote a Fenton-type reaction. Fenton-type reactions occur when reduced iron or copper are oxidized resulting in the formation of extremely toxic hydroxyl radicals (Asada, 1999; Halliwell and Gutteridge, 1999). To prevent the formation of hydroxyl radicals, photosynthetic cells have two primary enzyme systems for scavenging H_2O_2; ascorbate peroxidase and catalase (Asada and Takahashi, 1987; Kato, et al., 1997; Asada, 1999). Ascorbate peroxidase is associated with the chloroplast and is part of an elaborate scavenging system involving the coordination of multiple antioxidants (eg. ascorbate and glutathione) to prevent direct damage to photosynthesis (Foyer, et al., 1997; Takeda, et al., 1997; Asada, 1999). Catalase is typically localized in the microbody (peroxisome or glyoxisome), where H_2O_2 is generated during both the β-oxidation of fatty acids and the reactions of photorespiration (Stabenau, 1984; Pastori and del Rio, 1994; Kato, et al., 1997).

My research sought to compare the physiological implications of exposure to either UV-A or UV-B (independently) on photosynthetic processes and antioxidant activity in *Dunaliella* sp. The possible role played by inter-thylakoid β-carotene accumulation as a naturally existing blue light/UV filter was examined with respect to photosynthetic and antioxidant responses.

MATERIALS AND METHODS

Culture and extraction procedures

Dunaliella bardawil Ben-Amotz and Avron and *D. salina* were obtained from the University of Texas Culture Collection (UTEX 2538 and UTEX 200, respectively),

and cultures were grown in medium Gg containing 1.5M NaCl as described by Jahnke (1999). Air was bubbled through the cultures at a rate of 150-200 ml min^{-1} for a 150 ml culture. Cultures were grown in 38 X 200 mm tubes (either Pyrex or quartz) submerged in a water bath maintained at 26°C. The walls of the bath were made of UV-transmitting Plexiglas. Photosynthetically active radiation (PAR) used during culture growth phase was provided by a bank of Cool-White fluorescent lamps on one side of the water bath at 150 μmol m^{-2} s^{-1} ("high light"; HL). "Low light" (LL; 35 μmol m^{-2} s^{-1} PAR) was produced with neutral density wire screens placed between the lamp and culture tube. UV-A radiation was supplied by four lamps (2 General Electric F15T8 BLB and 2 WIKO F15T8 BLB) lamps on the opposite side of the bath, so the flux densities of PAR and UV radiation could be varied independently. UV-B was obtained from two Westinghouse Sunlamps (emission peak of 310 nm). Cultures were exposed to ultraviolet radiation for 24 hours in the experimental tanks with 10 mL harvested each hour for 0-3 hours and then at 24 hours of exposure to perform enzyme assays. **Figure 1.1** depicts the wavelength distribution of incident radiation within the cultures tubes for PAR, UV-A, and UV-B bulbs (Jahnke, 1999). The irradiance of UV-A at the surface of the culture tubes was approximately 110 μmol m^2 s^{-1} (36.1 W m^{-2}). Three different irradiance levels of UV-B were obtained using potassium chromate solutions around quartz culture tubes. The quartz culture tubes were 38 mm outer diameter and were placed within larger quartz tubes (60 mm inner diameter). The space between the inner and outer tubes (11 mm diameter) was filled with an aqueous K_2CrO_4 solution of known concentrations (0.25, 0.50, and 1.0

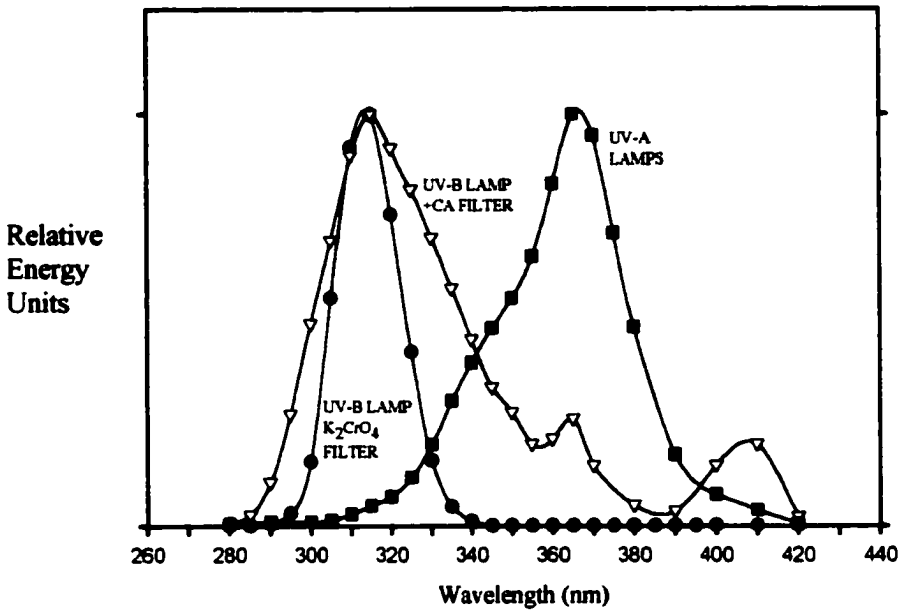

Fig. 1.1. Wavelength distribution of UV radiation incident on cultures. UV-B radiation was supplied by one Westinghouse FS20 Sunlamp filtered through either one layer of cellulose acetate (CA) (open triangles) or K_2CrO_4 (closed circles) as described in Material and Methods. UV-A radiation (closed squares) was produced by four lamps (2 General Electric 15T8 BLB and 2 WIKO 15T8 BLB). Radiation was measured with spectroradiometer fiber optics probe placed within culture tube *in situ*. Peak wavelengths normalized for comparison. (For photon flux densities, see Table 1). Visible radiation (PAR) was produced by four daylight fluorescent lamps, (spectrum not shown).

mM) at pH > 7.0. The chromate anion strongly absorbs both UV-A and UV-C, but not UV-B (**Fig. 1.1**). In addition, control cultures were established using UV blocking plastic film which absorbs all radiation below 395 nm (from Edmund Scientific, Barrington, NJ, type G39,426). The 1.0 mM K_2CrO_4 filter was determined to provide a UV-B exposure condition which was the threshold of photosynthetic pigment bleaching and cell death, and thus, was used for all UV-B experiments (**Table 1.1**). The wavelength distribution of all growth radiation was measured *in situ* with a spectroradiometer (International Light model 1700/760 D/783, Newburyport, MA) with a 2 nm bandpass and a fiber-optics probe calibrated by International Light. The

Table 1.1. Ultraviolet photon flux density within culture tubes and growth response of *Dunaliella bardawil* from one UV-B lamp (Westinghouse FS20 Sunlamp) with cellulose acetate or chromate filters.

UV Treatment	Cellulose Acetate	UV flux density, (μmol m^{-2} s^{-1})	(W m^{-2})	Growth rate, div. d^{-1}	Pigment bleaching
UV-B					
0 mM K_2CrO_4	-	10.8	4.15	0	yes
0 mM K_2CrO_4	+	9.2	3.54	0	yes
0.25	-	6.2	2.38	0	yes
0.50	-	3.3	1.27	0	yes
1.0	-	1.9	0.73	0.6	no
UV-A	-	110	36.1	0.6	no

Ultraviolet photon flux density measured *in situ* with the ferrioxalate chemical actinometer as described by Hatchard and Parker (1956). UV-B lamp: Westinghouse FS20 Sunlamp. Filters: one layer cellulose acetate surrounding the culture tube (to remove UV-C) or a solution of K_2CrO_4 of 11mm thickness surrounding the culture tube (to remove UV-C and UV-A; see Material and Methods, Fig. 1). Determined after 72 hours of exposure (26°C, 150 μmol m^{-2} s^{-1} PAR, air bubbled through cultures). A LiCor LI 185D radiometer was used to determine PAR flux densities. Similar results were obtained with *D. salina.*

photon flux density (400-700 nm) was measured with a Li-Cor LI-185 D quantum radiometer. Ultraviolet photon flux density was measured in situ with a ferrioxalate UV chemical actinometer (Hatchard and Parker, 1956). The actinometer fluid was placed within the culture tubes to reproduce the UV exposure geometry of the cultures.

Culture growth rates were measured as changes in absorbance at 687 nm with a Spectronic 21 spectrophotometer. All pigment concentrations were determined by the equations of Lichtenthaler (1987; **Table 1.2**). Extracts for enzyme and protein analysis were prepared by washing cells in 3.0 ml of isoosmotic glycerol followed by bench centrifugation at 1,200 x g (top speed). The pellet was resuspended in 1.0 ml hypoosmotic glycerol (0.5M) in 50mM phosphate buffer (pH 7.0), ultrasonicated for

15 sec., and microfuged at top speed for 2 min. The supernatant was stored on ice for protein, catalase, and ascorbate peroxidase analysis. Protein analysis was performed using the Coomasie-Blue method described by Bradford (1976).

Physiological measurements

Photosynthesis rates were measured under light limited and light saturated conditions at 25°C using Rank Brothers Clark-type oxygen electrodes and techniques described by Del Rio, et al. (1977). Clark-type oxygen electrodes calibrated to 25°C were used to record rates of photosynthetic oxygen evolution at five PAR irradiances: 50, 100, 250, 500, and 1000 μmol m^{-2} s^{-1}. Fv/Fm ratios were measured with an Opti-Science OS500 modulated fluorometer (Tyngsboro, MA). Samples containing approximately 3.0-4.0 μg chlorophyll/ml were equilibrated in darkness for 5 min. before Fv/Fm measurements were performed. In order to measure Fv/Fm, far-red light is initially pulsed through dark adapted cells fully oxidizing electron acceptors on the reducing side of PSII, prior to saturation.

The absorptance of HL and LL cells was determined with a 20 cm diameter integrating sphere (model 70491, Oriel Instruments, Stratford, CT). UV and visible radiation was supplied by fiber optics cable with a Xenon-arc source (model 66011, Oriel Instruments). Cell cultures containing equal chlorophyll *a* concentration were placed in a quartz cuvette within the sphere and non-absorbed radiation was measured with an International Light spectroradiometer. The equation of Öquist et al. (1978) was used to calculate the absorptance: I_o-I/I_o-I_d where, I_o = the spectral photon flux density in the empty sphere; I = the spectral photon flux density in the sphere with the

cell sample; and, I_d = the spectral photon flux density after the insertion of a black body of the same surface area as the algal sample.

Extracts were assayed spectrophotometrically to detect changes in ascorbate peroxidase activity (Nakano and Asada, 1981). Extracts for ascorbate and glutathoine assays were made by extracting from a pellet (10 ml of culture centrifuged 3 min. at 1,200 x g) with 1 ml solution containing 25 µmol perchloric acid, 12 µmol oxalic acid, 0.1 µmol diethylenetriamine pentaacetic acid, 15 mg metaphosphoric acid in 40% v/v methanol. Following 5 seconds sonication, the extract was microfuged for 2 min at 11,000 x g. Part of the supernatant was neutralized in the presence of 16 mM dithiothreitol for 10 minutes to reduce all DHA to ascorbate, and another fraction of the supernatant was retained as 'reduced' ascorbate. Each pair of samples were diluted with HPLC mobile phase and measured by HPLC with electrochemical oxidation at 0.7V as described by Behrens and Madere (1987). The oxidized ascorbate (i.e. DHA) is taken as the difference between the total ascorbate pool and the reduced ascorbate. The remainder of the extract was saved for determination of total glutathione using the method of Griffith (1980).

To examine how UV radiation affected photosynthetic parameters and antioxidant enzyme activity (response variables), randomized complete block, split plot two-way ANOVAs were performed using UV treatments as the main blocks, exposure time as subplots, and species as the blocking factor. When appropriate, Tukey-Kramer HD multiple comparison tests were utilized to compare means within treatments or between treatment combinations (Zar, 1999). Significance was established as $p \leq 0.05$.

RESULTS

Photosynthetic effects

Significant photobleaching occurred within all cells exposed to UV-B (using only cellulose acetate to block cultures from residual UV-C) within 24 hours, except when the 1.0 mM potassium chromate shield was utilized (**Table 1.1**). The concentration of ions within the liquid shield to be used for experimentation was determined based on the threshold of UV-B damage to chloroplast pigments (determined to be 1.0M potassium chromate solution).

Cultures of *D. bardawil* HL accumulated significantly more β-carotene than either *D. bardawil* LL or *D. salina* HL cultures (**Table 1.2**). The ratio of carotenoids to chlorophyll in *D. bardawil* HL was 1.11 ± 0.08 (g:g), as compared to *D. bardawil* LL and *D. salina* HL ratios of 0.26 and 0.29 ± 0.02, respectively. Pigment values for *D. bardawil* LL and *D. salina* HL were statistically the same ($p \leq 0.05$). The accumulation of β-carotene in *D. bardawil* HL cultures provided a dramatic increase

Table 1.2. Comparison of pigment:protein and carotenoid:chlorophyll (car:chl) ratios in *D. bardawil* HL, *D. bardawil* LL, and *D. salina*. Data are mean values (± Standard Error) of (*n*) replicate experiments. PAR intensity data indicates the growth conditions of cultures tested.

Sample	PAR intensity $\mu mol\ m^{-2}\ s^{-1}$ ($W\ m^{-2}$)	Car:Chl (g:g)	Chl:Protein (mg:g)	Car:Protein (mg:g)
D. bardawil HL	150 (32.7)	1.11±0.08 (*n*=7)	14.2±1.0 (*n*=7)	15.9±1.9 (*n*=7)
D. bardawil LL	35 (7.6)	0.26±0.02 (*n*=7)	17.1±0.57 (*n*=7)	4.51±0.36 (*n*=7)
D. salina	150 (32.7)	0.29±0.02 (*n*=4)	17.0±1.9 (*n*=4)	4.80±0.18 (*n*=4)

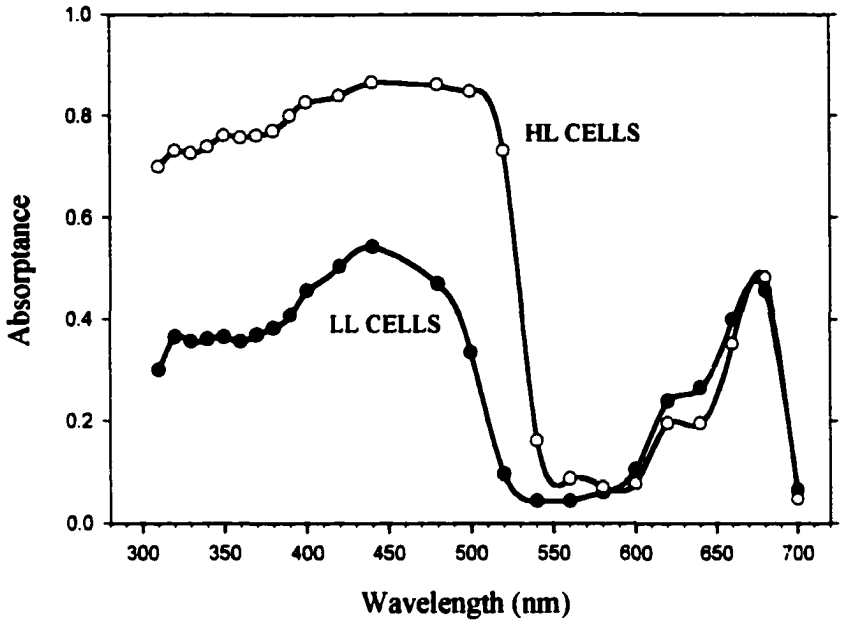

Fig. 1.2. Absorptance spectrum of intact cells of *D. bardawil* HL (150 μmol photons m^{-2} s^{-1}), *D. bardawil* LL (35 μmol photons m^{-2} s^{-1}), , and *D. salina* HL (150 μmol photons m^{-2} s^{-1}). Cultures contained equal chlorophyll *a* concentrations (absorptance normalized at 660 nm). Intact cells were placed within an integrating sphere (Oriel type), and, using a fiber optics probe, non-absorbed radiation was measured by a spectroradiometer. Equation of Ogren, et al. (1978) used to determine absorptance.

in the absorbance of blue and UV wavelengths as compared to either *D. bardawil* LL or *D. salina* HL cells (**Fig. 1.2**).

Following exposure to UV-B, there was no significant difference for Fv/Fm values in any of the cultures tested over a 24 hour period (**Fig. 1.3B**). UVA, however, did have a significant effect ($p \leq 0.05$) quantum yield of PSII fluorescence, as Fv/Fm values decreased dramatically (especially for *D. bardawil* LL and *D. salina*) within three hours for all of the cultures tested (**Fig. 1.3A**). It should be noted that *D. bardawil* HL (high β-carotene) samples exhibited significantly less damage than either *D. bardawil* LL or *D. salina*. The accumulation of β-carotene in *D. bardawil*

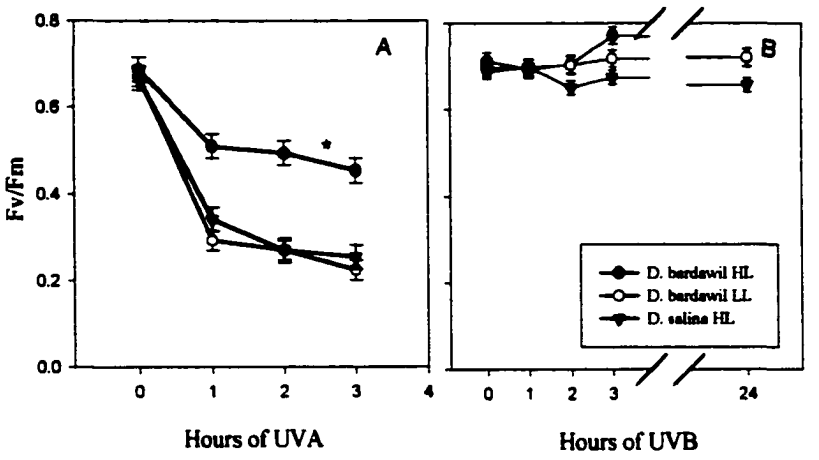

Fig. 1.3. Comparison of the change in Fv/Fm for *D. bardawil* HL, *D. bardawil* LL, and *D. salina* following exposure to UV-A (Graph A) and UV-B (Graph B). There is no statistical difference ($p \leq 0.05$) between any samples following exposure to UV-B, while *D. bardawil* HL was significantly different (*) from *D. bardawil* LL and *D. salina* following exposure to UV-A (which both exhibited significant decreases in Fv/Fm ratios within 3 hours).

HL appears to be correlated to photosynthetic protection following short-term exposure to UV-A (0-3 hours; **Fig. 1.5**).

Exposure to UV-A also elicited a significantly greater response than UV-B with respect to the ratio of light limited to light saturated photosynthesis (i.e. P_{100}:P_{500}, **Fig. 1.4A**). Again, both *D. salina* and *D. bardawil* LL exhibited a significant decrease after only 3 hours of exposure (about 80% and 90%, respectively; **Fig. 1.4A**). Interestingly, *D. bardawil* HL showed no significant change in ratios following 3 hours of exposure to UV-A. Following the UV-B treatment, there was no significant change observed for P_{100}:P_{500} ratios for any of the cultures tested over a 24 hour period (**Fig. 1.4B**). As the proportion of carotenoids to chlorophyll increases in *D. bardawil*, there is significantly reduced damage to both Fv/Fm values and light limited to light saturated photosynthetic ratios following UV-A exposure (**Fig. 1.5**).

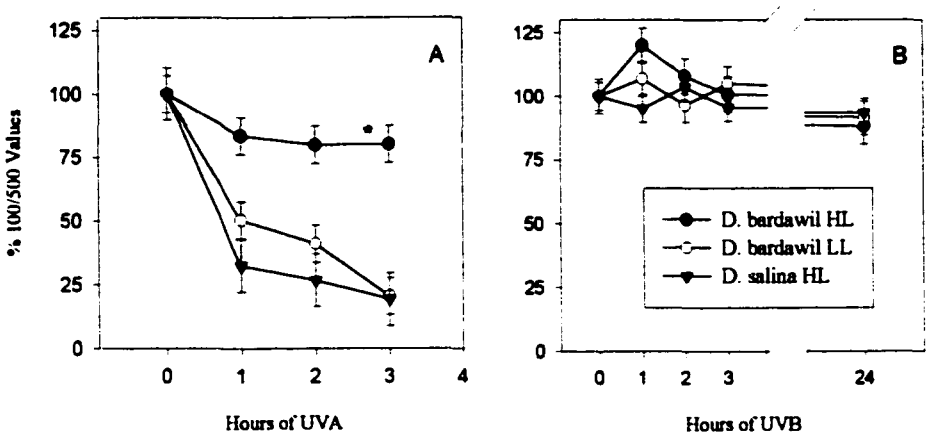

Fig. 1.4. Comparison of the percent change (from control) in light limited to light saturated photosynthesis ratios for *D. bardawil* HL, *D. bardawil* LL, and *D. salina* HL followng exposure to UV-A (Graph A) or UV-B (Graph B). There is no statistical difference ($p \leq 0.05$) between any samples following exposure to UV-B, while *D. bardawil* HL was significantly different (*) from *D. bardawil* LL and *D. salina* following exposure to UV-A (both had significant decreases in Fv/Fm within 3 hours).

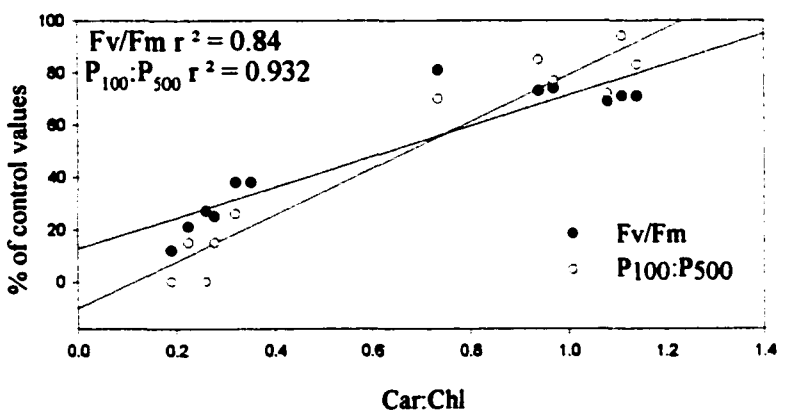

Fig. 1.5. Linear regression analysis of carotenoid accumulation (expressed as carotenoid:chlorophyll ratios) in *D. bardawil* and % of control Fv/Fm and light limited to light saturated photosynthetic ratios (P_{100}:P_{500}) following exposure to UVA for 3 hours.

Antioxidant enzyme effects

Exposure to pure UV-B did not have any significant effect on the activity of ascorbate peroxidase in any of the samples tested (**Fig. 1.6B**). Following exposure to UV-A, however, ascorbate peroxidase activity increased most dramatically in *D. bardawil* HL, while *D. bardawil* LL culture was intermediate (**Fig. 1.6A**). During the first three hours of UV-A exposure, *D. salina* actually exhibited a decrease in enzyme activity, and although activity did increase following 24 hours of exposure, it was the lowest overall rate of the three cultures tested. It should also be noted that *D. bardawil* HL exhibited a greater initial rate of enzyme activity (prior to UV-A exposure) than either *D. bardawil* low or *D. salina* cultures. Exposure to UV-A resulted in significant increases in the total ascorbate pool for *D. bardawil* LL, while *D. bardawil* HL exhibited smaller, but statistically significant, increases following

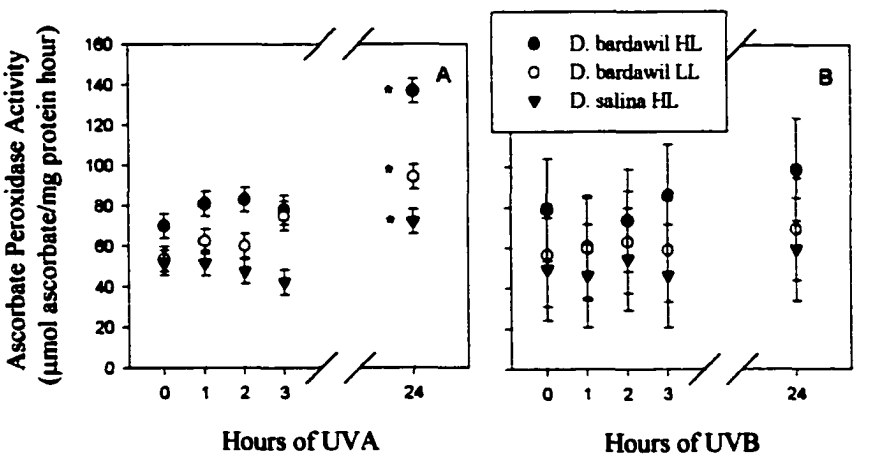

Fig. 1.6. Comparison of the change in ascorbate peroxidase activity for *D. bardawil* HL, *D. bardawil* LL, and *D. salina* following exposure to UV-A (Graph A) and UV-B (Graph B). There is no statistical change in ascorbate peroxidase activity following exposure to UV-A between any samples, while UV-A effects are statistically significant for all three test groups (*).

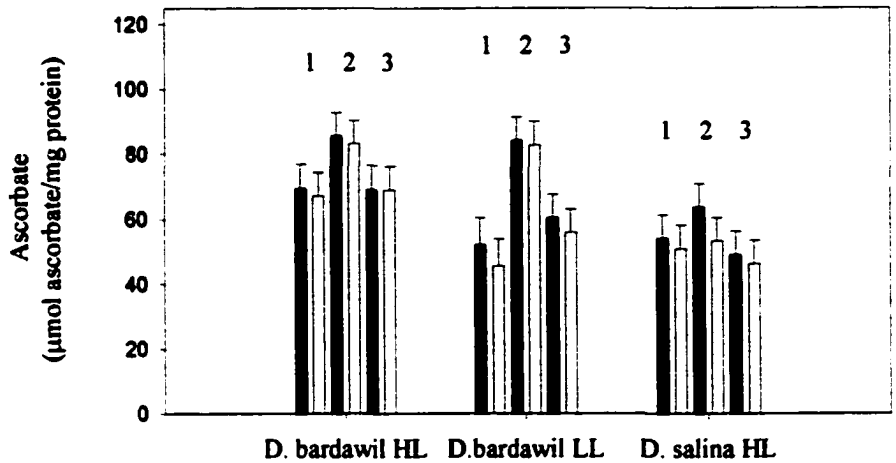

Fig. 1.7. Comparison of total (black bars) and reduced (white bars) ascorbate concentrations for *D. bardawil* HL, *D. bardawil* LL, and *D. salina* HL following exposure to either UV-A or UV-B for 24 hours. Numbers represent UV exposure treatments: 1-Control cells (no UV), 2-UV-A exposed cells, and 3-UV-B exposed cells. There were significant ($p \leq 0.05$) increases in both total and reduced ascorbate following exposure to UV-A in *D. bardawil* HL and LL, and both were significantly different from *D. salina*HL.

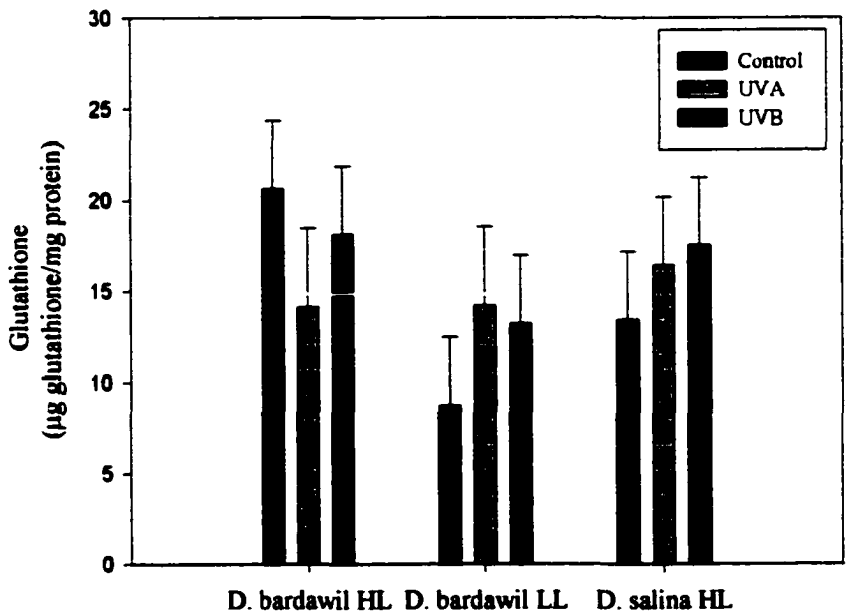

Fig. 1.8. Comparison of the change in total glutathione concentration following exposure to either UV-A or UV-B. There is no significant ($p \leq 0.05$) difference between cultures of *D. bardawil* and *D. salina*, or following exposure to UV-A or UV-B treatments for 24 hours.

exposure (**Fig. 1.7**). There was no significant change in total ascorbate content for any species following exposure to UV-B. In addition, no change in the total or reduced ascorbate pool was observed for *D. salina* following exposure to UV-A or UV-B. In all of the samples tested, reduced ascorbate comprised greater than 85% of the total pool. There was a significant decrease in glutathione concentrations in high PAR cultures of *D. bardawil* following exposure to UV-A, while low PAR cultures exhibited a significant increase in glutathione following exposure to UV-A or UV-B (**Fig. 1.8**). The high and low PAR cultures of *D. bardawil* were significantly different from each other; however, they were not significantly different from *D. salina*

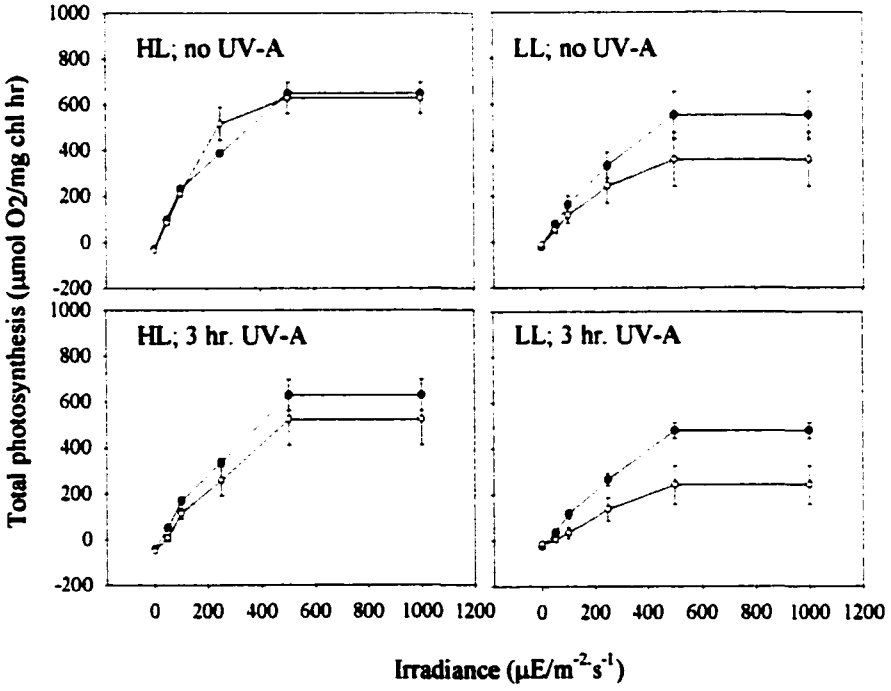

Fig. 1.9. Comparison of high and low PAR cultures of *D. bardawil* placed into UV-A for 0-3 hours, both screened (open circles) and unscreened (closed circles) to modulate PAR intensity. The treatments on the graphs are as follows: High PAR (HL; 150 µmol photons $m^{-2} s^{-1}$), no UV-A (same intensity PAR for both); Low PAR (LL; 3), no UV-A, High PAR (HL), 3 hours UV-A, (same PAR), and Low PAR (LL), 3 hours UV-A.

cultures. No significant change was observed following exposure to UV-A or UV-B in cultures of *D. salina*.

Exposure to UV-A in conjunction with increased PAR in LL cells of *D. bardawil* resulted in a significant decrease in P_{max} (rate of maximum photosynthesis as measured by oxygen evolution; **Fig. 1.9**). The decrease in P_{max} was only observed when cells were exposed to an increased level of PAR while simultaneously being exposed to UV-A. No difference in P_{max} was observed in HL cultures of *D. bardawil* exposed to UV-A when PAR levels are not increased.

Table 1.3. Unweighted experimental irradiances and total dose for each treatment group and weighted irradiances using the generalized higher plant weighting function of Caldwell, et al. (1971; *) and the chloroplast photoinhibition function of Jones and Kok (1966; **).

UV Treatment	Irradiance (Wm^{-2})	Dose (kJm^{-2})	Weighted Irradiance* (Wm^{-2})	Weighted Irradiance** (Wm^{-2})
UV-B + K_2CrO_4	0.73	63.1	4.46×10^{-6}	4.25×10^{-5}
UV-B	3.54	305.9	7.32×10^{-5}	3.00×10^{-4}
UV-A	36.1	3119.0	NA	2.34×10^{-5}

UV-B spectral treatments were compared with and without the use of the potassium chromate filter (1.0 mM K_2CrO_4); both UV-B treatments were filtered using cellulose acetate (CA). Measurements were normalized to 1.0 at 290 nm. Dosage was determined based on 24 hour exposure periods.

Table 1.3 shows the UV irradiance for each treatment along with the total dose for the duration of the experiment. In addition, the biologically effective irradiance for each treatment was calculated using weighting functions for generalized higher plant photosynthetic damage (Caldwell, et al., 1971) and chloroplast photoinhibition (Jones and Kok, 1966).

DISCUSSION

When plants are exposed to ultraviolet light, whose photons are of much higher energy than that of visible photons, a series of reactions may occur to dissipate the excess energy in the cell. Through these reactions and others, radical forms of oxygen are often created which may then cause significant, irreversible damage to important metabolic processes within the cell (such as lesions in DNA and inhibition of photosynthesis). Specifically, reactive oxygen intermediates (ROI's) are formed during the conversion of oxygen to water creating free-radical intermediates and the relatively stable oxidant hydrogen peroxide. Shaish, et al. (1993) have suggested that photosynthetically produced oxygen radicals are involved in triggering massive β-carotene accumulation in *D. bardawil*. The work of Jahnke (1999) supports this because low levels of UV-A irradiance triggered the accumulation of β-carotene in *D. bardawil*. The effects of both UV-A and UV-B exposure will be discussed with respect to photosynthetic parameters and antioxidant enzyme activity.

Photosynthetic parameters

It has been suggested that ambient levels of UV-A and UV-B irradiance do not cause significant decreases in the growth of photosynthetic organisms, as acclimation allows for photosynthetic adjustments. Allen, et al., (1998) have stated that photosynthesis is only directly inhibited following exposure to high UV-B irradiance and involves the loss of soluble Calvin cycle enzymes and adaxial stomatal closure, rather than damage to the light reaction apparatus. This review goes on to suggest that

even under extreme UV-B intensities the photosynthetic apparatus may be protected through acclimation, and the primary areas of research interest should be plant development and associated processes.

Decreases in cell growth following exposure to high irradiance UV-B are most likely correlated with damage to DNA and photobleaching (**Table 1.1**). There is a significant amount of information regarding the effects of UV-B on DNA, specifically with regard to the formation of photoproducts (mainly cyclobutyl-type dimers; Franklin and Forster, 1997). Through the formation of these photoproducts, the potential exists for lethal errors during DNA transcription and synthesis or during RNA translation (Franklin and Forster, 1997; Jeffrey and Mitchell, 1997). In addition to dimers, UV-B may directly induce photohydrates in DNA by light-catalyzed addition of a water molecule to a pyrimidine base (Jeffrey and Mitchell, 1997). Thus, decreases in the growth rate of algal cells exposed to high irradiance UV-B are most likely a result of the formation of dimers and other photoproducts in conjunction with DNA, as well as photobleaching.

Ultraviolet-A irradiance has been correlated to a decrease in cell growth; however, damage is a result of indirect effects, as opposed to UV-B-related direct damage. Jeffrey and Mitchell (1997) have reported that the majority of the aerobic lethal response to UV-A radiation is a result of photooxidative processes and the generation of reactive oxygen intermediates. Formation of eeactive oxygen species in the presence of UV-A may lead to a wide array of DNA damage including the modification of bases, strand breaks, alkali-labile sites, and DNA-protein crosslinks. The formation of single strand breaks (the main mechanism implicated in cell death)

are induced in cells exposed to UV-A radiation (365nm). This process is sensitized by oxygen and can be reduced by irradiation in the presence of radical scavengers (Jeffrey and Mitchell, 1997; Peak and Peak, 1990). Decreases in cell growth of *Dunaliella* sp. following long-term UVA exposure was most likely a result of the indirect effects of reactive oxygen damage to DNA. During short-term exposure, antioxidant enzymes are capable of preventing damage, however, as the duration of exposure increases, damaged DNA can not be repaired and cells succumb to radical formation and death.

While high irradience UV-B appears to cause significant damage to cells with respect to DNA and cell death, there does not appear to be a significant effect on PSII, specifically, Fv/Fm (**Fig. 1.3B**). This finding is consistent with past research that suggests PSII is not the primary site of damage to photosynthesis following exposure to UV-B (Franklin and Forster, 1997; Nogues and Baker, 1995). Nogues and Baker (1995) examined the effects of high irradience UV-B exposure (8 times the summer maximum) on photosynthetic parameters in pea plants and found that Fv/Fm ratios fell only after the photosynthetic capacity of the leaf had been substantially reduced. The work of Lesser (1996a,b) further substantiated this observation in the dinoflagellates *Prorocentrum micans* and *Symbiodinium bermudense* grown at an irradiance of 120 μmol $m^{-2}s^{-1}$ and then exposed to UV-B for 21 days. Lesser found that variable fluorescence was unchanged, however, significant decreases in RubisCO activity and photosynthetic capacity were observed following UV-B exposure. Franklin and Forster (1997) suggest that the reduction of P_{max} without changes in maximum quantum yield is unknown in PAR photoinhibition and may be a

diagnostic feature of UV damage, consistent with the findings of this research (**Fig. 1.9**).

In contrast to the effects of UV-B, UV-A exposure appeared to have a significant effect on Fv/Fm ratios in both species of *Dunaliella* tested, with the least damage occurring in samples of *D. bardawil* with a high accumulation of β-carotene (**Fig. 1.3A**). There has been substantial research that demonstrates the correlation between UV-A and decreases in both photosynthetic efficiency and Fv/Fm in algae. Dring, et al (1996) examined a broad range of red macroalgae exposed to only PAR + UVA and found that UV-A had a greater effect on Fv/Fm in conjunction with PAR than UV-B. In addition, Hermann, et al. (1995) found that there was no difference in the reduction of Fv/Fm of *Ulva laetevirens* when wavelengths under 320nm were removed from natural irradiance. This again suggests that the majority of Fv/Fm damage is a result of UV-A wavelengths, as damage still occurs even when UV-B wavelengths are removed.

Hirosawa and Miyachi (1983) have shown that inhibition of the Hill reaction is mediated through a chromophore absorbing in the UV-A region, thus the absorption (filtration) of UV-A in the high β-carotene cells may lend selective protection from photosynthetic damage by preventing the inhibition of the Hill reaction and electron transport. Past research has demonstrated that the globules of β-carotene accumulated by *D. bardawil* under stress conditions effectively screen blue light and offer significant protection against photoinhibition (Jahnke, 1999; Ben-Amotz, et al., 1989). Furthermore, *in vivo* analysis of high β-carotene *D. bardawil* showed a two- to four-fold increase in absorbance of wavelengths between 350-400nm (**Fig. 1.2**). It has

been suggested that this may provide the cell with significant screening of middle- and long-wavelength UV-A radiation.

Theoretically, increases in the ratio of light limited to light saturated photosynthesis (P_{100}:P_{500}) are indicative of damage to RubisCO (and thus, carbon fixation), while decreases in the ratio are indicative of damage to electron transport efficiency. Changes in this ratio are correlated to both UV exposure and PAR irradience, due to photoinhibition. At low PAR intensities, the rate of photosynthetic activity is proportional to the amount of light intercepted by the photosynthetic apparatus (Geider and Osborne, 1992). Results from this study indicate that damage to photosynthesis increased when cells were simultaneously exposed to UV-A and PAR intensities greater than those used in culture (**Fig. 1.9**). Decreases in the ratio of light limited to light saturated photosynthesis were observed when cells were placed in UV-A + increased PAR intensities, as would be expected if there was a disruption in the efficiency of electron transport. Many studies have identified a correlation between PAR irradience and photoinhibition via damage to the D1 and/or D2 reaction center proteins during periods of excess excitation energy absorption (Maxwell, et al., 1997; Ohad, et al., 1994; Aro, et al., 1993).

Low β-carotene cells of *D. bardawil* and *D. salina* exhibited a significant decrease in the ratio of light limited to light saturated photosynthesis, while high β-carotene cells of *D. bardawil* exhibited little change in this ratio (**Fig. 1.4A and B**). The decrease in light limited to light saturated photosynthetic ratios are indicative of a increase in the formation of reactive molecules as electron transport efficiency is compromised. This is consistent with the hypothesis of Shaish, et al. (1993) that

connects the formation of active oxygen species to the induction of β-carotene in *D. bardawil*. The hypothesis states that at high irradiances, photosynthetic electron transport in algae with little accumulation of β-carotene is impaired, most likely a result of PSII damage. Overexcitation of damaged photosynthetic components leads to the enhanced formation of reactive oxygen species which, in turn, activates the transcription of β-carotene biosynthesis genes. Once the genes are activated, β-carotene is accumulated in order to screen the light-harvesting complexes against overexcitation and prevent photooxidation. This proposed mode of action for β-carotene accumulation in *D. bardawil* is consistent with the findings presented in this study. Shaish found that *D. bardawil* with high levels of β-carotene was fully protected from photoinhibition when exposed to high irradiance blue light. High accumulation of β-carotene also is responsible for a significant absorption of UV-A wavelengths, preventing damage to photosynthesis using the same mode of action (i.e. high energy blue light absorption) as observed in high PAR conditions correlated to photoinhibition. The major blue/near-UV screening effect of ß-carotene was measured by Loeblich (1982). She compared the action spectra of photosynthesis (as oxygen evolution) in *Dunaliella* with high vs. low ß-carotene. The low carotene cells had significant photosynthesis excited by narrow band radiation throughout the range 330 to 500nm. Photosynthetic excitation rates were reduced by 45-55% at the wavelengths 330-500nm in cells with high concentrations of β-carotene (an indication of the effectiveness of blue light absorption by accumulated β-carotene).

In addition, UV-A has been linked to inhibition of alternative respiration in leaves and algae. Goyal and Tolbert (1991) found that exposure to UV-A resulted in a reduced capacity of alternative respiration in *Chlamydomonas*, and recovery occurred within 24 hours following the removal of the UV treatment. One function of alternative respiration may be the dissipation of excess energy in order to prevent the formation of superoxide within the mitochondria. If the alternative oxidase mechanism is damaged, there is the potential for the accumulation of reactive oxygen species

Exposure to UV-B appeared to cause only a fraction of the damage to photosynthetic activity as UV-A and cells appeared to recover from damage within 24 hours of exposure (**Fig. 1.4B**). Short-term exposure to UV-B resulted in a small increase in the ratio of light limited to light saturated photosynthesis, indicative of damage to RubisCO. There has been a wide array of information linking UV-B exposure to RubisCO damage through either direct or indirect mechanisms (Allen, et al., 1998; Lesser, 1996a,b; Asada and Takahashi, 1987). Direct damage may occur following UV-B exposure by aromatic amino acid absorption, while indirect damage to RubisCO has been attributed to reactive oxygen formation within the cell.

Antioxidant enzyme activity

Past research has suggested that in response to stress conditions and subsequent radical formation, plants have formed a complex network of antioxidant enzymes capable of scavenging reactive molecules before damage can occur. The evidence has indicated that exposure to both high PAR and UV-A irradiance leads to increased

formation of reactive oxygen within the chloroplast, as judged by the increase in both ascorbate peroxidase activity and the total ascorbate pool within the cells (**Figs. 1.6 and 1.7**). Following 24 hours of exposure to UV-A, the greatest increase in ascorbate peroxidase activity was observed in the high PAR + UV-A exposed cells of *D. bardawil*. These results are consistent with expectations as *D. bardawil* accumulates β-carotene in response to stress; however, the accumulated globules are not believed to actually scavenge reactive molecules or act as a nonphotochemical quencher.

There has been considerable research providing a link between physiological stress, such as high PAR, UV irradiance, and temperature extremes, and increased antioxidant activity (Foyer and Mullineaux, 1994). Shick, et al. (1995) found that the activities of antioxidant enzymes were considerably higher in shallow-water coral zooxanthellae than in those found in deeper water, an indication that high irradiance conditions result in the formation of reactive oxygen molecules and trigger antioxidant responses. Malanga and Puntarulo (1995) examined the effects of UV-B exposure on antioxidant content in *Chlorella vulgaris* and summarized that the presence of UV irradiance induces both the superoxide dismutase and ascorbate peroxidase enzyme systems. Although the antioxidant system found in photosynthetic organisms is highly effective, research suggests that as stress conditions are compounded, these systems alone may not provide enough protection to prevent irreversible damage (Lesser, 1996a,b). Furthermore, although increases in antioxidant activity were not sufficient to prevent radical formation under extreme stress conditions, the addition of exogenous active oxygen scavengers (i.e. ascorbate and catalase) did help to mitigate some photosynthetic damage. This further substantiates

that damage to photosynthesis was, in fact, related to the formation of reactive oxygen species within the chloroplast. The increase in ascorbate peroxidase activity following UV-A exposure (with the highest activity in high β-carotene samples) suggests that the inter-thylakoid accumulation of β-carotene in *D. bardawil* is not scavenging radicals (acting as a nonphotochemical quencher) but merely acting as a blue light filter to mitigate photodamage.

Observed trends for ascorbate indicate that, once again, short-term UV-A exposure is correlated to increased radical formation (as observed by the dramatic increase in the ascorbate pool in low β-carotene cells of *D. bardawil*), while UV-B exposure is not (**Fig. 1.7**). In addition, the accumulation of β-carotene appears to lend selective protection to the chloroplast through screening of the blue/UV-A wavelengths responsible for instigating the formation of reactive oxygen molecules. In all cases, the majority of the ascorbate pool was found to be in a reduced form, an indication that observed increases in the total pool were related to a heightened need for radical scavenging, particularly following UV-A exposure. The induction of glutathione synthesis has been characterized as a cellular response that increases the availability of cycling antioxidants and buffers against the accumulation of H_2O_2 *in vivo* (Noctor, et al., 2000). It has been suggested that as reactive oxygen species accumulate cells respond by synthesizing excess ascorbate and glutathione to scavenge ROS and prevent metabolic damage. Following exposure to UV-A, glutathione content increased substantially in low PAR cultures of *D. bardawil* and high PAR cultures of *D. salina* with an increase noted following exposure to UV-B, as well (**Fig. 1.8**). High PAR (i.e. high β-carotene) cultures of *D. bardawil* did not

exhibit an accumulation of glutathione following exposure to UV-A or UV-B. It may be suggested that the screening of blue/UV radiation by accumulated β-carotene and the filtration of excessive irradiance reduced the formation of reactive oxygen species, *in vivo.*

The results suggest exposure to ultraviolet-A (but not ultraviolet-B) radiation directly affects photosynthesis, observed as a loss of photosystem II electron transport efficiency (and increased radical formation). This research proposes that the accumulated β-carotene in *Dunaliella bardawil* prevents UV-related photosynthetic damage through blue-light/ultraviolet-A absorption and is supported by trends observed for antioxidant enzyme responses.

CHAPTER II

THE EFFECTS OF SPECTRAL QUALITY AND QUANTITY ON CATALASE ISOZYME ACTIVITY IN *DUNALIELLA* SPP.

ABSTRACT

The effect of a variety of spectral conditions on the activity of the enzyme catalase was examined in the single-cell green alga *Dunaliella bardawil*. In addition, cells with high levels of accumulated interthylakoid β-carotene were compared to cells with little accumulation to observe the role of β-carotene as a blue light filter to the enzyme catalase. Cells were cultured in high and low PAR environments (150 and 35 μmol photons m^{-1} s^{-1}, respectively). Cultures were exposed to ultraviolet-A (UV-A; 320-400 nm) + PAR, ultraviolet-B (UV-B; 290-320 nm) +PAR, or high red (wavelengths > 590 nm) or blue wavelengths (390-540 nm; 150 μmol photons m^{-1} s^{-1}), independently, for 24 hour test periods. Catalase activity was measured following culture in various spectral exposure conditions to monitor long-term catalase responses. Following growth in one of the spectral growth conditions, cells were exposed to UV-A or UV-B to observe short-term responses of catalase activity (and the photoprotective ability of chloroplast-associated β-carotene). Results indicate that the association of β-carotene with chloroplasts in *D. bardawil* has little protective function for the enzyme catalase, and appears to provide protection maximally to the chloroplast. Effects of ultraviolet exposure on catalase activity appeared to be species

specific and associated with isoform stimulation/inactivation. In addition, isoform activity appeared to be correlated to the ratio of oxygen to carbon dioxide. This study indicates that exposure to a variety of light intensities and wavelengths elicits different responses of catalase isoforms (and thus, enzyme activity) depending on the particular species of *Dunaliella.*

INTRODUCTION

The formation of reactive oxygen intermediates (ROI) in aerobic organisms is an inevitable process, potentially resulting in a wide array of metabolic damage to cells. ROI's are formed during the conversion of oxygen (O_2) to water (H_2O) creating free radical oxygen intermediates, as well as, the relatively stable oxidant, hydrogen peroxide (H_2O_2; Scandalios, 1997). Once formed, ROI's have the potential to cause morphological, physiological, biochemical and molecular damage within the cell. There are two types of reactions that generate H_2O_2 in peroxisomes, mitochondria, and chloroplasts: 1) the univalent reduction of oxygen to form superoxide radicals followed by a dismutation into H_2O_2 and O_2 and, 2) divalent reduction of molecular O_2 enzymatically (i.e. glycolate oxidase; Ishikawa, et al., 1993). Hydrogen peroxide is a relatively stable oxidant and not highly reactive, however, the transition-metal catalyzed Fenton reaction can form a highly reactive hydroxyl radical ($^{\cdot}OH$; Asada and Takahashi, 1987; Halliwell and Gutteridge, 1985). Hydroxyl radicals may also be formed from H_2O_2 exposure to ultraviolet radiation and subsequent homolytic fission (removal/addition of a single electron). The hydroxyl radical can then cause damage

to proteins, fatty acids and bilayer lipid membranes resulting in an array of physiological damage within the cell (Scandalios, 1997). In addition, UV related formation of $OH^{\cdot}$ from H_2O_2 appears to affect DNA, most frequently causing single strand breaks and DNA protein cross links (Halliwell and Gutteridge, 1985). Since H_2O_2 can freely diffuse across cell membranes there must be a coordinated effort by several enzymes to scavenge it completely.

The peroxisomes are one of the main sources of H_2O_2 generation in photosynthetic cells. During photorespiratory reactions, H_2O_2 is generated through the divalent reduction of dioxygen by glycolate oxidase. Most cyanobacteria and algae have carbon concentrating mechanisms, consisting of an extremely effective enzyme/pump system to minimize photorespiration in cells. In addition to photorespiration, H_2O_2 is also produced within peroxisomes during the β-oxidation of fatty acids. In order to protect the cell from damage related to the production of H_2O_2 peroxisomes contain the scavenging enzyme, catalase (Scandalios, 1994).

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6; CAT) is a tetrameric, heme-containing enzyme, capable of acting in two ways. First, at low concentrations, catalase may act peroxidatively to oxidize a hydrogen donor, and second, at high concentrations, catalase rapidly degrades H_2O_2 acting as both a hydrogen donor and acceptor (Scandalios, 1994; Aebi, 1984). Catalase exhibits a high K_m for H_2O_2 as a substrate, and although is it not possible to saturate the enzyme with reasonable substrate concentrations (up to 5M H_2O_2), concentrations above 0.1M H_2O_2 have been shown to cause the rapid inactivation of catalase (Aebi, 1984).

Research has suggested that catalase synthesis may be sensitive to light, with prolonged exposure resulting in photoinactivation. Cheng, et al. (1981) found that light corresponding to the maximal absorbance of the heme site of catalase (405nm) was the most effective in the inactivation of the enzyme. Feierabend and Engle (1986) examined photoinhibition of catalase, *in vivo*, and found that although natural levels of light intensity did not cause photoinactivation of catalase, amplified light intensity and red light did. Research has also suggested that isozymes of catalase exhibit different degrees of photoinactivation. Grotjohann, et al. (1997) isolated catalase isozymes CAT2 through CAT8 and found that forms CAT2 - CAT5 were less photosensitive than other forms of the enzyme. Polidoros and Scandalios (1997) examined the difference between the light independent expression of *Cat1* genes and light dependent expression of *Cat 2* and *3* genes in maize and found that all three genes did, in fact, exhibit different responses to light and circadian patterns. In addition to inactivation due to prolonged light exposure, catalase is sensitive to a variety of environmental stresses including O_2:CO_2 ratio (Butow, et al., 1994), pH (Butow, et al., 1994), temperature (Volk and Feierabend, 1989), ultraviolet light (Malanga and Puntarulo, 1995), and salt (Streb and Feierabend, 1995).

It is the goal of this research to identify the number of catalase isoforms present in the unicellular green alga *Dunaliella*, as well as, the effect of various spectral conditions on catalase activity (and the relative activity of isoforms). Spectral conditions compared include high and low intensity photosynthetically active radiation (PAR), high intensity red and blue light, ultraviolet-a (UV-A), and ultraviolet-b (UV-B). In addition, the ability of chloroplast-associated β-carotene

(accumulated by *D. bardawil* under stress conditions) to filter blue/UV wavelengths (and prevent catalase inactivation) is examined.

MATERIALS AND METHODS

Culture and extraction procedures

Dunaliella bardawil Ben-Amotz and Avron and *D. salina* were obtained from the University of Texas Culture Collection (UTEX 2538 and UTEX 200, respectively), and cultures were grown in medium Gg containing 1.5M NaCl as described by Jahnke (1999). Air was bubbled through the cultures at a rate of 150-200 ml min^{-1} for a 150 ml culture. Cultures were grown in 38 X 200 mm tubes (either Pyrex or quartz) submerged in a water bath maintained at 26°C. The walls of the bath were made of UV-transmitting Plexiglas. Growth photosynthetically active radiation (PAR) was provided by a bank of Cool-White fluorescent lamps on one side of the water bath at 150 μmol m^{-2} s^{-1} ("high light"; HL). "Low light" (LL; 35 μmol m^{-2} s^{-1} PAR) was produced with wire screens placed between the lamp and culture tube. High red (all wavelengths > 590 nm) and high blue light (390-540 nm, 462 nm peak), both 150 μmol m^{-2} s^{-1} photons, exposure conditions were created using plastic filters. UV-A radiation was supplied by four lamps (two General Electric F15T8 BLB and two WIKO F15T8 BLB) on the opposite side of the bath, so the flux densities of PAR and UV radiation could be varied independently. UV-B was obtained from one Westinghouse Sunlamp (emission peak of 310 nm). Cultures were exposed to ultraviolet radiation for 24 hours in the experimental tanks with 10 mL harvested each

hour for 0-3 hours and then at 24 hours of exposure to perform enzyme assays. Figure 1.1 depicts the wavelength distribution of incident radiation within the cultures tubes for UV-A and UV-B bulbs (Jahnke, 1999). The irradiance of UV-A at the surface of the culture tubes was approximately 110 μmol $m^2 s^{-1}$. Three different intensities of UV-B were obtained using potassium chromate solutions around quartz culture tubes. The quartz culture tubes were 38 mm outer diameter and were placed within larger quartz tubes (59 mm inner diameter). The space between the inner and outer tubes was filled with an aqueous $KCrO_4$ solution of known concentrations, 1.0 mM, at pH > 7.0. The chromate anion strongly absorbs both UV-A and UV-C, but not UV-B (**Chapter I, Fig. 1.1**). In addition, control cultures were established using UV blocking plastic film (from Edmund Scientific, Barrington, NJ, type G39,426). The wavelength distribution of all growth radiation was measured in situ with a spectroradiometer (International Light model 1700/760 D/783, Newburyport, MA) with a 2 nm bandpass and a fiber-optics probe calibrated by International Light. The photon flux density (400-700 nm) was measured with a Li-Cor LI-185 D quantum radiometer. Ultraviolet photon flux density was measured in situ with a ferrioxalate UV chemical actinometer. The actinometer fluid was placed within the culture tubes to reproduce the UV exposure geometry of the cultures.

Culture growth rates were measured as changes in turbidity at 687 nm with a Spectronic 21 spectrophotometer. All pigment concentrations were determined by the equations of Lichtenthaler (1987). Extracts were prepared by washing cells in 3.0 ml of isoosmotic glycerol. The pellet was resuspended in 1.0 ml hypoosmotic glycerol (0.5M) in 50mM phosphate buffer (pH 7.0), ultrasonicated for 15 sec., and

microfuged at top speed for 2 min. The supernatant was stored on ice for protein, catalase, and ascorbate peroxidase analysis. Protein analysis was performed using the Coomasie-Blue method described by Bradford (1976).

Enzyme measurements

Biochemical analysis:

The absorbance spectrum of catalase was determined using a Perkin-Elmer lambda 3B UV/VIS double beam spectrophotometer. Changes in catalase activity were measured as changes in oxygen evolution using Clark oxygen electrodes and techniques described by Del Rio, et al. (1977). Electrodes were calibrated at 25^0C and3.0mL of 50mM TRIS (pH 7.8) was added to the sample chamber. Dissolved oxygen was removed from the buffer by bubbling with nitrogen gas and then the chamber was sealed. 100μL of H_2O_2 (ca. 3% solution) was added to the chamber with a Hamilton syringe and the oxygen level was monitored for 3 min. to determine a stable rate. Once a stable rate was established, 30μL of extract was added using a Hamilton syringe and the oxygen level was again monitored for 3 min. to determine the rate of catalase activity (reported in μmol O_2/mg protein hour).

Extracts for ascorbate and glutathione assays were made by extracting a pellet (from 10 ml of culture) with 1 ml solution containing 25 μmol perchloric acid, 12 μmol oxalic acid, 0.1 μmol diethylenetriamine pentaacetic acid, 15 mg metaphosphoric acid in 40% v/v methanol. Following 10 seconds sonication, the extract was microfuged for 2 min at 11,000g. Part of the supernatant was neutralized in the presence of 16 mM dithiothreitol for 10 minutes to reduce all DHA to ascorbate, and another fraction of the supernatant was retained as 'reduced' ascorbate. Each pair of samples were diluted with HPLC

mobile phase and measured by HPLC with electrochemical oxidation at 0.7V as described by Behrens and Madere (1987). The oxidized ascorbate (i.e. DHA) is taken as the difference between the total ascorbate pool and the reduced ascorbate. The remainder of the extract was saved for determination of total glutathione using the method of Griffith (1980).

To examine how UV radiation affected catalase and antioxidant substrate concentrations (response variables), randomized complete block, split plot two-way ANOVAs were performed using irradiance treatments as the main blocks and species as the blocking factor. When appropriate, Tukey-Kramer HD multiple comparison tests were utilized to compare means within treatments or between treatment combinations (Zar, 1999). Significance was established as $p \leq 0.05$.

Isoform detection (PAGE analysis):

Extracts were prepared for catalase and superoxide dismutase isozyme visualization as previously described and then samples were loaded on 7.5% (catalase) or 10% (SOD) mini-gels for nondenaturing polyacrylamide gel electrophoresis (PAGE) using the BIO-Rad mini-gel apparatus. Standard Lamelli conditions were applied for buffering solutions and gels were run constant at 125V for approximately 1.5 hours. Following electrophoresis, gels were removed and washed in three changes of water for about 45 min. total (15 min. each) on a slow shake. In order to stain for catalase isozymes, the gels were placed into 0.007% H_2O_2 and shaken for 10 min. Following H_2O_2 incubation, the gel was rinsed briefly and placed into a 1.0% solution of ferric chloride/potassium ferricyanide (made from recently made 2.0% stock solutions of each) for 10 min. to visualize isoform bands

(Woodbury, et al., 1970). Isozymes of SOD were visualized by soaking the gel (20 min.) in 2.5 mM nitroblue tetrazolium, briefly rinsing, followed by soaking in 0.028M EDTA, 0.0028 mM riboflavin, and 0.036 M phosphate buffer (pH 7.8) for 15 min. (Beauchamp and Fridovich, 1971).

RESULTS

Cultures were grown in high PAR (150 μmol m^{-2} s^{-1}; both *D. bardawil* and *D. salina*) and then placed into low PAR (35 μmol m^{-2} s^{-1}), high red, and high blue (both 150 μmol m^{-2} s^{-1}) exposure conditions for 24 hours. It was observed that high light

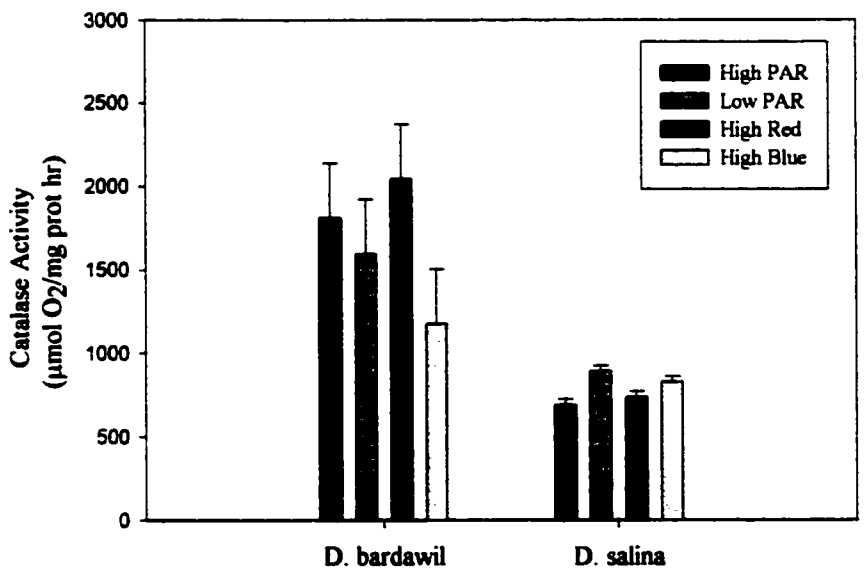

Fig. 2.1. Comparison of the change in total catalase activity following exposure to a variety of spectral treatments for 24 hours. Cultures were grown in high PAR, low CO_2 conditions and then exposed to low PAR, high red, and high blue photons for 24 hours. Only low PAR cultures of *D. bardawil* were significantly ($p \leq 0.05$) different from the other light treatments, and *D. bardawil* was significantly different from *D. salina*.

(i.e. PAR, Blue and Red) exposure resulted in a significant increase in catalase activity as compared to low PAR cultures (**Fig. 2.1**) of *Dunaliella bardawil*. In contrast, *D. salina* exhibited significantly lower catalase activity than *D. bardawil* in any of the treatments applied (**Fig. 2.1**). There were no significant differences observed for catalase following exposure to any of the light treatments in *D. salina*.

All three cultures exhibited approximately a 20% increase in catalase activity within the first three hours of UV-B exposure (**Fig. 2.2A**) however, a similar increase was noted in control samples when compared to various UV-B filtration methods (**Fig. 2.3**). Cells were filtered from UV-B using either 1.0mM K_2CrO_4, cellulose acetate (CA), or UV blocking plastic film (from Edmund Scientific, Barrington, NJ, type G39,426; i.e. control). Significant ($p \leq 0.05$) decreases in catalase activity were noted for all cellulose acetate-filtered cultures within 24 hours. Significant decreases

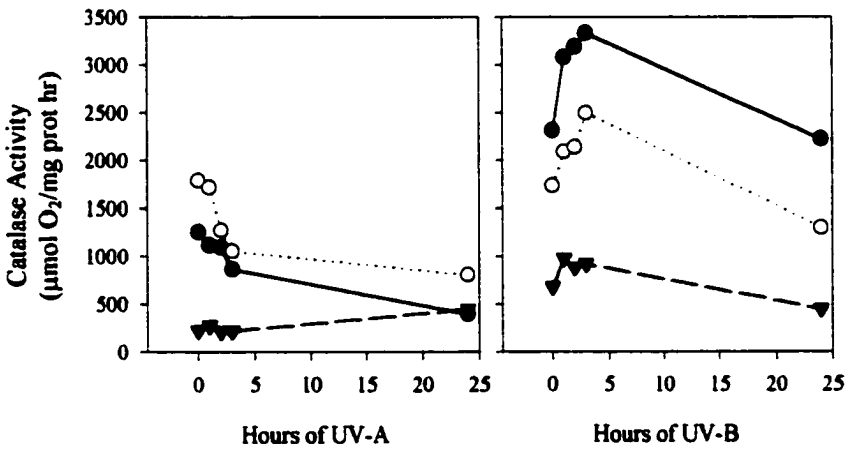

Fig. 2.2A and B. Comparison of the change in total catalase activity in *D. bardawil* HL (closed circles), *D. bardawil* LL (open circles), and *D. salina* (closed triangles) following exposure to either UV-A (Graph A) or UV-B (Graph B) for 24 hours. There is no statistically significant difference between either *D. bardawil* HL or LL cultures, while *D. salina* was significantly different from both in UV-A and UV-B experiments. Both *D. bardawil* cultures exhibited a significant decrease in catalase activity in the UV-A treatment but no significant change in UV-B, while there was no change in catalase activity for *D. salina*.

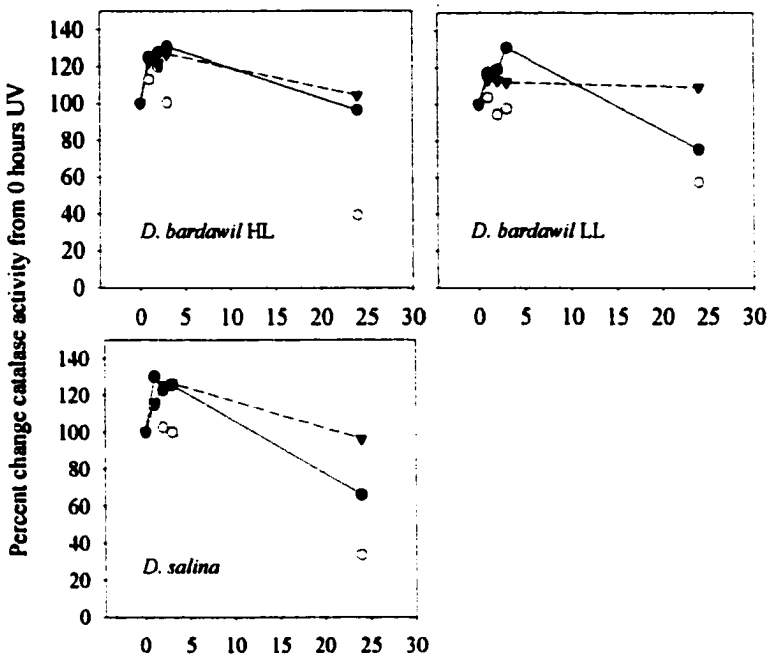

Fig. 2.3. Comparison of the effect of various UV-B filtration methods on catalase activity in *D. bardawil* HL, *D. bardawil* LL, and *D. salina* (HL) following 24 hours of exposure. Change in activity is expressed as the percent change from 0 hours of UV-B exposure. Cells were filtered from UV-B using either 1.0mM K_2CrO_4 (closed circles), cellulose acetate (CA; open circles), or UV-blocking plastic (Control; closed triangles). Significant ($p \leq 0.05$) decreases in catalase activity were noted for all cellulose acetate-filtered cultures within 24 hours. Significant decreases of catalase in K_2CrO_4-filtered cultures of *D. bardawil* LL and *D. salina* occurred, as well.

of catalase in K_2CrO_4-filtered cultures of *D. bardawil* LL and *D. salina* occurred, as Statistically, there was no significant difference in catalase activity between either *D. bardawil* culture (HL or LL), while *D. salina* was significantly different from *D.bardawil*. Effects of UV-A appear to be species specific, as well, as there was a significant decrease in catalase activity for both *D. bardawil* cultures (>50% enzyme inhibition) while *D. salina* exhibited a marked increase in enzyme activity following exposure to UV-A (**Fig. 2.2A**). Ultimately, following 24 hours of UV-A exposure, all three sample groups exhibited the same values for catalase activity.

PAGE analysis confirms the results of the biochemical assays performed, indicating that catalase isoform effects from various light and UV treatments are species specific. PAGE detection revealed three isoforms of catalase present in *D. bardawil* (**Fig. 2.4**), while only two forms of the enzyme were detected in *D. salina* (**Fig. 2.5**). Cultures of *D. bardawil* acclimated to either high PAR or high blue light

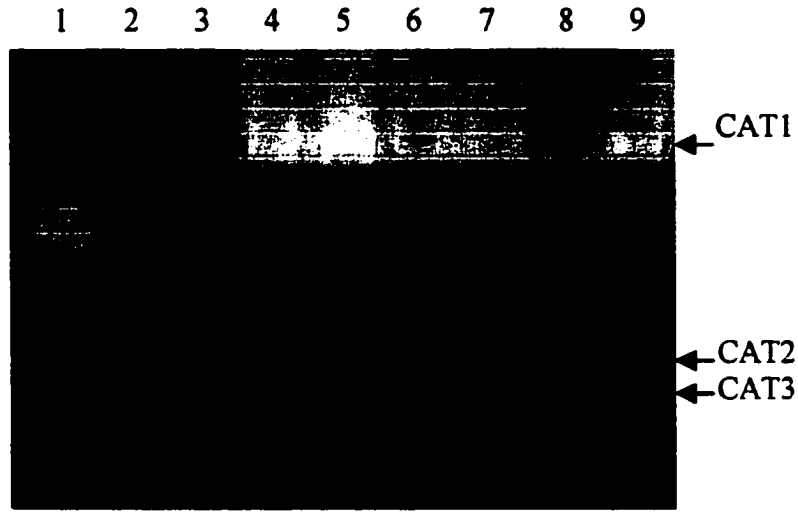

Fig. 2.4 . Visualization of catalase isozymes in *D. bardawil* under various light treatments using non-denaturing polyacrylamide gel electrophoresis (PAGE). Arrows indicate the location of various isoforms (3 forms total). Lanes 1-3 contain catalase standards (from bovine erythrocytes) in a final concentration of 1.0, 0.50, and 0.25 U/ml, respectively. The following treatments were used: Lane 4 - high PAR (CAT1 and 3); Lane 5 - low PAR (CAT1 and 2); Lane 6 - high red light (CAT1, 2, and 3); Lane 7 - high blue light (CAT1 and 3); Lane 8 - UV-A (CAT1); Lane 9 - UV-B (CAT1). Cells were aerated with low CO_2 (air) and placed into the various treatments for 24 hours.

conditions possessed active forms of CAT1 and CAT3, while low PAR cultures possessed active forms of CAT1 and CAT2. High red light acclimation resulted in the activation of all three isoforms in *D. bardawil*, with CAT3 exhibiting the greatest increase in isoform relative activity. Both UV-A and UV-B appeared to cause the inactivation of CAT2 and CAT3 in *D. bardawil*, with exposure to UV-A resulting

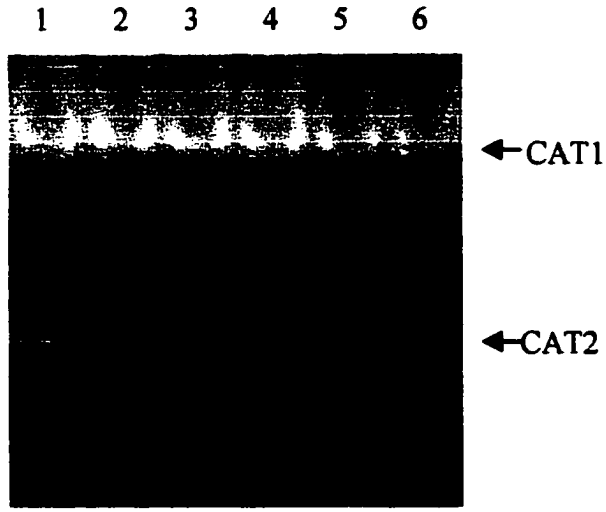

Fig. 2.5. Visualization of catalase isozymes in *D. salina* under various light treatments using non-denaturing polyacrylamide gel electrophoresis (PAGE). Arrows indicate the location of various isoforms (2 forms total). The following treatments were used: Lane 1 - high PAR (CAT1 and 2); Lane 2 - low PAR (CAT1 and 2); Lane 3 - high red light (CAT1 and 2); Lane 4 - high blue light (CAT1 and 2); Lane 5 - UV-A (CAT1); Lane 6 - UV-B (CAT1 and 2). Cells were aerated with low CO_2 (air) and placed into the various treatments for 24 hours.

Table 2.1. Comparison of catalase isozyme activity in *D. bardawil* and *D. salina* following exposure to various light treatments (high PAR, low PAR, high red light, high blue light, UV-A and UV-B) for 24 hours. Changes in relative activity of isozymes are indicated: (+) = increased activity; (-) = decreased activity.

Sample	High PAR	Low PAR	Red	Blue	UV-A	UV-B
D. bardawil (low CO_2)	CAT1 CAT3	CAT1 CAT2	CAT1 CAT2 CAT3(+)	CAT1 CAT3	CAT1(-)	CAT1
D. salina (low CO_2)	CAT1 CAT2	CAT1 CAT2	CAT1 CAT2	CAT1 CAT2	CAT1	CAT1 CAT2
D. bardawil (high CO_2)	CAT1 CAT2 CAT3	CAT1 CAT2(+) CAT3	CAT1 CAT2 CAT3	CAT1 CAT2 CAT3	CAT1(-)	CAT1 CAT2 CAT3
D. salina (high CO_2)	CAT1 CAT2(+)	CAT1 CAT2	CAT1 CAT2	CAT1 CAT2	CAT1	CAT1 CAT2

in a dramatic decrease in the activity of CAT1, as well. **Table 2.1** summarizes the effects of the various light treatments on catalase isozymes in *D. bardawil* and *D. salina* cultured in both high and low CO_2 conditions.

PAGE analysis performed on cultures of *D. bardawil* and *D. salina* using high CO_2 confirmed that there are 2 forms of the enzyme in *D. salina* and 3 in *D. bardawil* (**Figs. 2.6 and 2.7**). Results for test cultures of *D. bardawil* demonstrated that exposure in *Dunaliella salina* possessed only two forms of the enzyme, CAT1 and CAT3, which appeared to be active in all of the treatments, other than UV-A (**Fig. 2.7**). There did not appear to be any effect on enzyme isoform activity following acclimation to high PAR, low PAR, high red light, high blue light, or UV-B in *D. salina*, while the UV-A treatment resulted in the inactivation of CAT3. Interestingly,

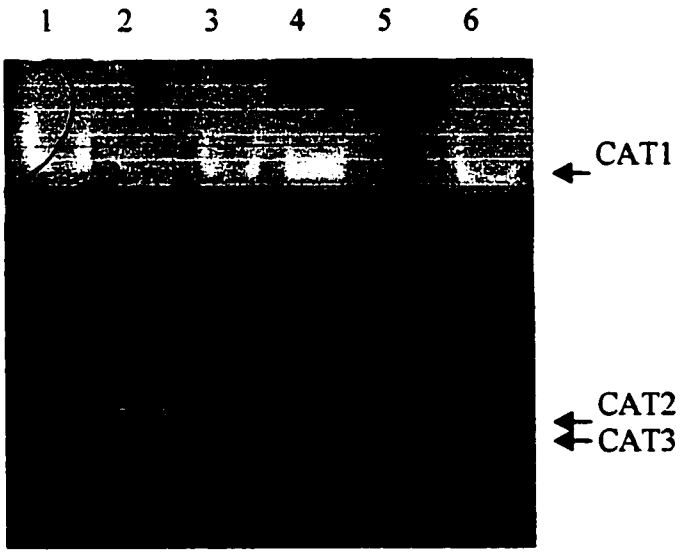

Fig. 2.6. Visualization of catalase isozymes in *D. bardawil* under various light treatments (and high CO_2) using non-denaturing polyacrylamide gel electrophoresis (PAGE). Arrows indicate the location of various isoforms (3 forms total). The following treatments were used: Lane 1 - high PAR (CAT1, 2 and 3); Lane 2 - low PAR (CAT1, 2 and 3); Lane 3 - high red light (CAT1, 2 and 3); Lane 4 - high blue light (CAT1, 2 and 3); Lane 5 - UV-A (CAT1); Lane 6 - UV-B (CAT1, 2 and 3). Cells were aerated with high CO_2 and placed into the various treatments for 24 hours.

unlike *D. bardawil*, the activity of the primary form of the enzyme (ie. CAT1) actually appeared to increase following exposure to UV-A radiation. These results are consistent with the trends exhibited by biochemical assays performed on total catalase activity within these two species of *Dunaliella*. Exposure to the various light treatments in conjunction with increased CO_2 aeration produced the same results as those found for low CO_2 aerated cultures (**Fig. 2.7**).

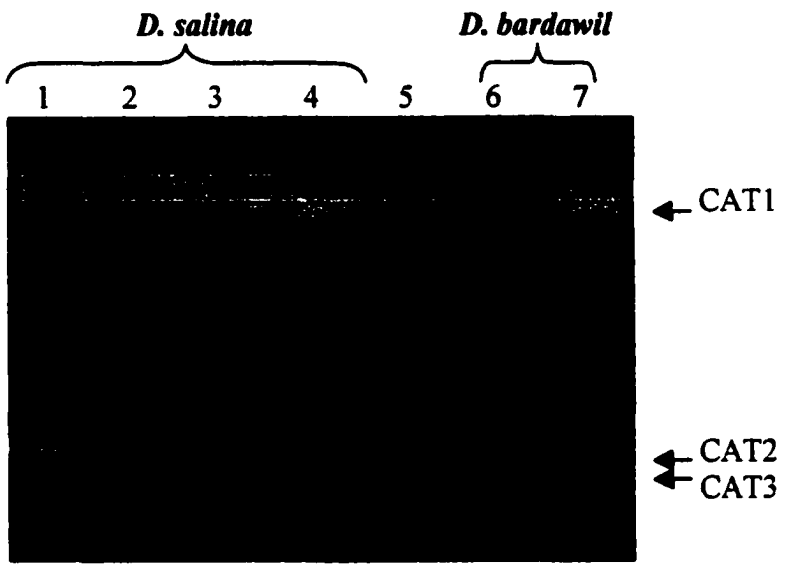

Fig. 2.7. Visualization of catalase isozymes in *D. salina* and *D. bardawil* under various light treatments (and high CO_2) using non-denaturing polyacrylamide gel electrophoresis (PAGE). Arrows indicate the location of various isoforms (2 forms total for *D. salina*; 3 forms total for *D. bardawil*). The following treatments were used: Lane 1 - high PAR (CAT1 and 2); Lane 2 - high red light (CAT1 and 2); Lane 3 - high blue light (CAT1 and 2); Lane 4 - UV-A (CAT1); Lane 5 - UV-B (CAT1 and 2); Lane 6 - high PAR (CAT1, 2 and 3); Lane 7 - low PAR (CAT1, 2 and 3). Cells were aerated with high CO_2 and placed into the various treatments for 24 hours. *D. bardawil* served as a marker for isoform band location in *D. salina*.

PAGE analysis of superoxide dismutase isozymes revealed 4 forms of the enzyme in *D. salina* and 3 forms in *D. bardawil* (**Fig. 2.8**). There did not appear to be any effect on isozyme activity following exposure to UV-A or UV-B (2 intensities were

compared for UV-B: 1.9 μmol photons m^{-2} s^{-1} and 3.3 μmol photons m^{-2} s^{-1}) in either of the species tested.

Following exposure to the various light treatments (i.e. high PAR, low PAR, high red, and high blue) there was no significant change in the total ascorbate pool (total pool = oxidized + reduced ascorbate) or in the ratio of reduced to oxidized ascorbate

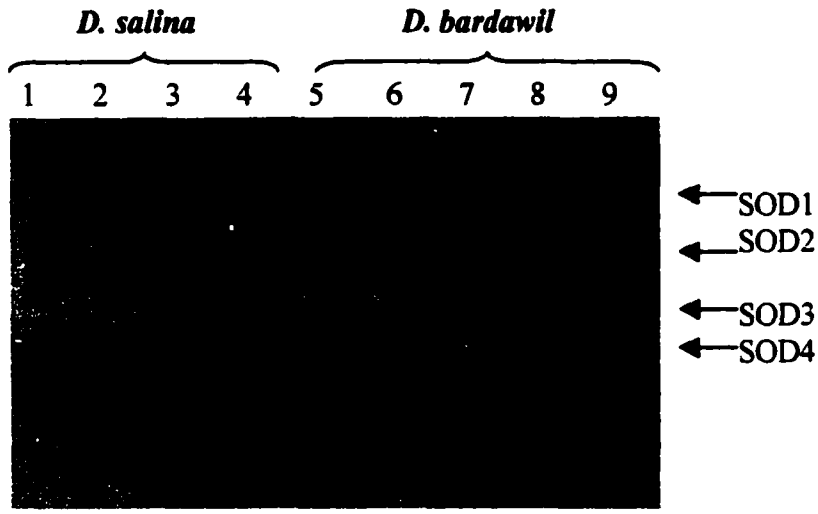

Fig. 2.8. Visualization of superoxide dismutase isozymes in *D. salina* and *D. bardawil* under various UV treatments using non-denaturing polyacrylamide gel electrophoresis (PAGE). Assays were run using 10% gels and staining techniques described by Beauchamp and Fridovich (1971). Arrows indicate the location of various isoforms (3 forms total for *D. bardawil*; 4 forms total for *D. salina*). The following treatments were used: Lane 1 - HL control; Lane 2 - UV-A exposure; Lane 3 - UV-B exposure (3.3 μmol photons m^{-2} s^{-1}); Lane 4 - UV-B exposure (3.3 μmol photons m^{-2} s^{-1}); Lane 5 - HL control; Lane 6 - HL + UV-A; Lane 7 - LL control; and Lane 8 - LL + UV-A. Cells were bubbled with air and placed into the various treatments for 24 hours.

except in the low light treatment (**Fig. 2.9**). Glutathione measurements following exposure to the various light treatments were similar to ascorbate, as the only significant decrease in the glutathione pool was observed in low light cells of *D. bardawil*. Glutathione concentrations increased significantly following exposure to both high PAR and high red light in *D. bardawil*, while there was no change in

glutathione concentrations for *D. salina* following experimental light treatments (**Fig. 2.10**).

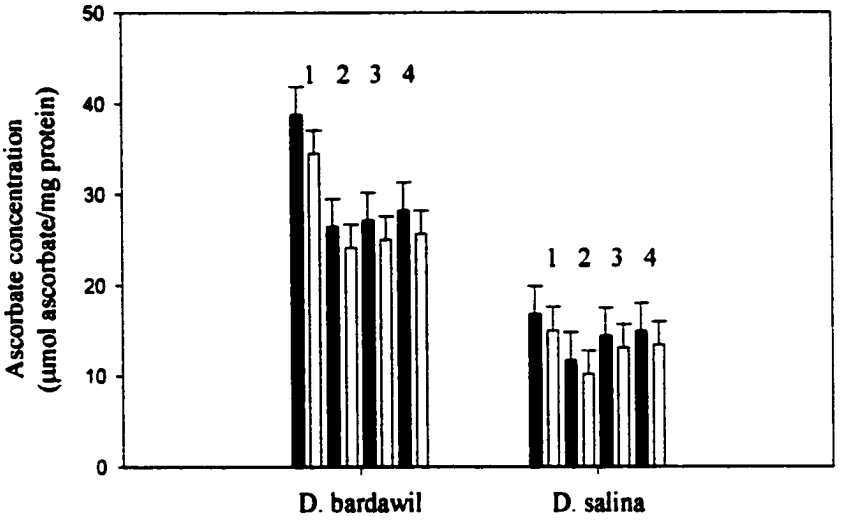

Figure 2.9. Comparison of total (black bars) and reduced (white bars) ascorbate concentrations in *D. bardawil* and *D. salina* following exposure to a variety of light treatments. Treatments are identified by number: 1 - high PAR (150 µmol photons m^{-2} s^{-1}), 2 - Low PAR (35 µmol photons m^{-2} s), 3 - High Red, and 4 - High Blue Light (both 150 µmol photons). Only low PAR cultures were significantly ($p \leq 0.05$) different from other light treatments for both *D. bardawil* and *D. salina*.

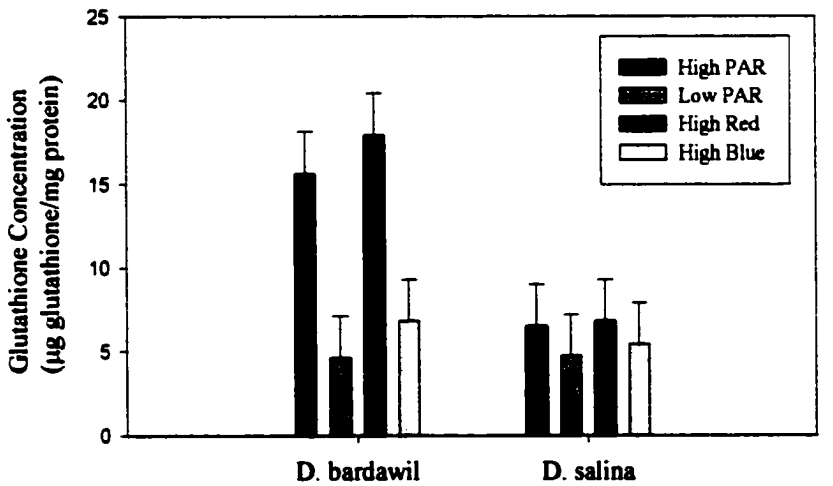

Fig. 2.10. Comparison of glutathione concentrations in *D. bardawil* and *D. salina* following exposure to a variety of light treatments (high PAR, low PAR, high red, and high blue) for 24 hours. Glutathione concentrations in high PAR and high red treatments were significantly ($p \leq 0.05$) greater than low PAR and high blue in *D. bardawil*, while there was no effect of treatment on *D. salina*. In addition, *D. bardawil* cultures were significantly different from *D. salina*.

DISCUSSION

Effects of spectral quality and quantity on antioxidants

Biochemical analysis of total catalase activity following exposure to a variety of light treatments indicates that exposure to high irradiance red light results in a significantly higher catalase activity in *D. bardawil*, than high blue, high PAR, or low PAR treatments (**Fig. 2.1**). PAGE analysis supports the findings of total catalase activity, as exposure to high red light (in conjunction with low CO_2) resulted in an obvious increase in the activity of the CAT3 isozyme (**Fig. 2.4**). *D. salina* exhibited a significantly lower constitutive catalase rate than *D. bardawil* in any light treatment, although activity does not appear to decrease following exposure to UV radiation (UV-A or UV-B). When the activity of *D. bardawil* does decrease following exposure to UV treatments, the total enzyme activity after 24 hours is essentially the same as that exhibited by *D. salina.*

In higher plants, exposure to high intensity red light has been correlated to increases in the ascorbate pool through the action of phytochrome signaling. Shigeoka, et al. (1979) found that blue light exposure resulted in an increase of reduced L-ascorbic acid in *Euglena*, however, red light had no effect (thus, an indication that there was no phytochrome action present). In *Dunaliella*, exposure to high PAR, high red, and high blue light equally effected ascorbate concentrations, while exposure to low PAR conditions resulted in a significantly smaller pool (**Fig. 2.9**). These findings are consistent with research that has demonstrated that the total

pool of ascorbate (reduced ascorbate, in particular) increases as light intensity increases (Smirnoff, 2000; Smirnoff and Pallanca, 1996; Smirnoff, 1995).

In contrast to ascorbate, the glutathione pool in *Dunaliella* increased following exposure to high PAR or high red light, with the most pronounced increase occurring in *D. bardawil* (**Fig. 2.10**). The induction of glutathione synthesis has been characterized as a metabolic strategy that increases the availability of cycling antioxidants and to buffer against the accumulation of H_2O_2 *in vivo* (Noctor, et al., 2000).

Furthermore, research suggests that when catalase activity is sufficient in the scavenging of H_2O_2, the redox state of the antioxidant pools recover (even though H_2O_2 levels remain elevated) and upregulation of glutathione synthesis does not occur (Noctor, et al., 2000). These findings are supported by catalase isoform activity (**Figs. 2.5 and 2.10**) and glutathione synthesis in *D. salina*, as there was no change in catalase isozyme activity following exposure to any radiation treatments (except UV-A), therefore, it would not be expected that glutathione synthesis would be induced. Although there were a variety of effects on the isozymes of *D. bardawil*, total catalase activity indicates that the overall rate of enzyme activity is not decreasing following exposure to any radiation treatment, except the UV-A treatment (**Figs. 2.1, 2.2 and 2.4**). As catalase activity decreases following exposure to UV-A, glutathione concentrations increase (**Chapter I, Fig. 1.8**) in low PAR cultures accompanied by increases in total ascorbate concentrations (**Chapter I, Fig. 1.7**). H_2O_2 accumulation in *Dunaliella* may possibly act as a signal for various antioxidant responses. When catalase is incapable of scavenging sufficient quantities of H_2O_2 to prevent metabolic

damage, cells accumulate both ascorbate and glutathione to increase the availability of cycling antioxidants and to prevent the formation of reactive oxygen species.

The role of H_2O_2 as a signaling agent has been well documented in a wide array of photosynthetic organisms. As a membrane-permeable molecule, H_2O_2 functions as a diffusible intercellular signal involved in the induction of a variety of stress defenses, including the elevation/inhibition of catalase (Morita, et al., 1999; Scandalios, et al., 1997; Prasad, et al., 1994) and ascorbate peroxidase (Morita, et al., 1999; Lappartient and Touraine, 1997; Pastory and Tripp, 1992). In addition, the accumulation of H_2O_2 has been observed in response to both UV radiation (Murphy and Huerta, 1990) and excess light (Karpinsky, et al., 1997).

The decrease in catalase activity noted in *D. bardawil* following UV-A treatment is likely related to light absorption of the heme moiety of the enzyme within the Soret band (405 nm; **Fig. 2.2**). When exposed to UV-B, there was a decrease in enzyme activity as well; however, the greatest decrease occurred following exposure to UV-B using only cellulose acetate to filter cell cultures (**Fig. 2.3**). Interestingly, no change in catalase activity was observed in *D. salina* following treatment with PAR and UV-A or UV-B, or high PAR, high red or high blue radiation (**Fig. 2.2**). These results are consistent with findings of PAGE analysis, as catalase isoforms appear to be unaffected by radiation treatments, other than UV-A. The invariable total rate of catalase in *D. salina* following UV-A exposure suggests that the primary isoform of the enzyme (i.e. CAT1) is compensating for activity decreases related to the loss of CAT2 activity. Like *D. salina*, the inactivation of catalase isoforms in *D. bardawil* does not appear to effect total catalase activity, except in the case of UV-A where

there was a significant loss of enzyme activity (both CAT2 and CAT3 are inactivated in low CO_2 conditions).

Photoinactivation of catalase isoforms

Previous research has indicated that the number of catalase isozymes present in a cell varies among photosynthetic organisms. Considerable research has been performed in order to characterize the various isoforms of catalase in higher plants, such as maize (Boldt and Scandalios, 1997; Polidoros and Scandalios, 1997), sunflower (Palomo, et al., 1999; Grotjohann, et al., 1997), and other economically important crops (Garcia, et al., 2000; Corpas, et al, 1997, Havir and McHale, 1990). Only limited research is available that characterizes catalase isoforms in algae. The lack of relevant research is most likely due to the absence of H_2O_2 formation during photorespiration in many species, due to the absence of glycolate oxidase (Kato, et al., 1997; Collen, et al., 1995). Whether from plant or animal tissue, however, catalase sensitivity has been demonstrated for a number of environmental stressors (or stimuli) that either enhance or inhibit isoform activities. The findings of the present research indicate that similar trends are observed in the microalgal species *Dunaliella* with respect to environmental effects on catalase isoform activation/inhibition. There appear to be two primary mechanisms for catalase isozyme inactivation: 1) direct inactivation of catalase through absorbance of excess radiation by the associated heme group (405 nm peak), and/or 2) indirect inactivation as a result of the photooxidative reactions of the chloroplast (and the formation of reactive molecules).

Grotjohann, et al. (1997) examined the *in vitro* photoinactivation of catalase isoforms in the cotyledons of sunflower and found that exposure to 405 nm was optimal for the inactivation of catalase (also known as the "soret band"). The research indicated that certain forms of the enzyme were more susceptible to photoinactivation than others, and was a result of reactive oxygen formation associated with the heme moiety. The varying degree of photosensitivity among isozymes assures a minimum level of protection when the intensity of radiation fluctuates over time. The CAT2 form of catalase found in *D. bardawil* was inactivated in either high PAR or high blue light conditions, as well as, following exposure to either UV-A or UV-B (**Fig. 2.4**). This inactivation was dependent on the ratio of CO_2 to O_2, as CAT2 was not inactivated (except in the presence of UV-A) when higher concentrations of CO_2 were used to aerate the cultures (**Fig. 2.6**). It may be suggested that the CAT2 form of catalase found in *D. bardawil* exhibits a high degree of sensitivity to blue/UV radiation related to the formation of reactive oxygen (under low CO_2 conditions) by either the heme moiety or through chloroplast photooxidative reactions (or both).

In addition to the observed inactivation of CAT2 in *D.bardawil* cultures grown under low CO_2:O_2 conditions, CAT3 was inactivated following the application of low PAR and UV (UV-A or UV-B) treatments, but again, only in low CO_2 conditions (**Fig. 2.4**). CAT3 activity was consistently enhanced by high red light exposure in low CO_2 *D. bardawil* cultures, a trend not observed in high CO_2 cultures. Polidoros and Scandalios (1997) have found that the induction of the *Cat3* circadian expression in maize is regulated by a chromophore (i.e. phytochrome) response, in conjunction with a blue/UV-A photoreceptor. Phytochrome absorbance of red (Pr) and far-red light

(Pfr) has been identified as an important regulatory mechanism in photosynthetic organisms. Absorption of red light transforms Pr to Pfr, which is considered the physiologically active form of the photoreceptor (Casal, 2000). It is quite possible that the response observed for *D. bardawil* CAT3 following exposure to red light may be a primitive expression of phytochrome regulation of catalase isozymes. Phytochrome-mediated responses have been observed in both higher plants (Casal, 2000; Lin, 2000; Casal, 1996) and algae (LeBlanc, et al., 1999; Wu, et al., 1997; Morand, et al., 1993; Kidd, et al., 1990), and appear to be involved in a complex redox regulatory network (Casal, 2000; Hermsmeier, et al., 1991; Shigeoka, et al., 1979)

In maize, Polidoros and Scandalios (1997) have found that there are three genes for catalase and all respond differently to light signals. One gene for catalase (*Cat1*) is light independent, the second is regulated transcriptionally by both light and UV exposure, and the third exhibits a transcriptionally regulated circadian rhythm. Circadian control of catalase isozymes has been extensively studied in a number of higher plant species (Lin, 2000; Somers, 1999; McClung and Kay, 1994). The induction of a circadian rhythm for the CAT3 form of catalase in maize has been detected in plants grown in either constant darkness or continuous light following exposure to UV radiation (290-400 nm; Boldt and Scandalios, 1997; Polidoros and Scandalios, 1997). In *Arabidopsis*, circadian rhythm has been identified as an important component of expression for genes encoding both chlorophyll-*a*/*b*-binding proteins (*CAB2*), as well as, catalases (specifically *Cat2* and *Cat3*). Miller, et al. (1995) found that *CAB2*-promoter activity was influenced by continuous exposure to

either blue or red wavelengths of light, an indication that photoreceptors are key components of gene regulation. Based on the initiation of circadian patterns, it has been suggested that regulation may be a function of photoreceptors and the role of UV as an environmental cue. More extensive research is required to determine the relationship between catalase gene regulation and light conditions in unicellular algae; however, this research suggests that within the species of *Dunaliella* alone there is great diversity with respect to the responses of the various catalase isoforms to both PAR and UV irradiance. Interestingly, these responses appear to be related to the formation of reactive oxygen species in low CO_2 conditions, particularly in *D. bardawil*.

Shang and Feierabend (1999) found that rye leaf catalase was inactivated following exposure to high irradiance red light, yet no sensitizer for red light was detected in isolated peroxisomes. It was suggested that the red light-inactivation of catalase was a result of photooxidative reactions initiated in the chloroplasts as this inactivation is oxygen dependent. The chloroplast-mediated inactivation of catalase was determined to provide a means for the detection of a redox signalling system of chloroplasts that was postulated to indicate overreduction of plastoquinones. The intensity of the red light used for the study was 800 μmol photons m^{-2} s^{-1}, while only 150 μmol photons m^{-2} s^{-1} were used in this research. It is possible that the intensity used with *Dunaliella* initiated the formation of ROS, yet, not to an extent that completely inactivated catalase. At low concentrations, radical formation has been found to initiate catalase activity, as appears to be the case with *D. bardawil* since the stimulation of CAT3 was dependent on the ratio of CO_2 to O_2.

Orendi, et al. (2001) examined the loss of stress-induced expression of CAT3 during leaf senescence in *Arabidopsis* as a function of oxidative stress. The enzyme activities of all superoxide dismutase (SOD) and ascorbate peroxidase (APX) isoforms remained unaffected following treatment with paraquat, ozone, high light, or UV-B, while CAT3 activity was enhanced. PAGE analysis of *D. bardawil* and *D. salina* was consistent with the findings of Orendi, as there was no visible change in isoform activity for SOD following exposure to UV-A or UV-B for 24 hours (**Fig. 2.8**). Orendi suggests that SOD activity does not appear to be a critical point in oxidative stress management in *Arabidopsis* senescing leaves, as severe paraquat treatments did not induce an increased level of SOD or APX activity, while catalase responses were quite varied.

Analysis of total and reduced ascorbate concentrations following exposure to high PAR, low PAR, high red light and high blue light are consistent with findings of Orendi, as well. There was little change in the ascorbate pool following exposure to the various light treatments. Although there was a significantly smaller pool consistently in LL cells, all high light treatments resulted in similar concentrations of ascorbate for both *D. bardawil* and *D. salina.* (**Fig. 2.9**). The lack of increased substrate concentrations for ascorbate is an indication that the intensity of blue and red light applied was not any more stressful than high PAR to photosynthetic processes. These results indicate that catalase isozyme responses in *Dunaliella* are most likely a result of direct effects on the heme moiety of the enzyme, perhaps in conjunction with some increased chloroplastic production of reactive oxygen as light intensity increases, or blue/UV-A regulatory signalling.

Production of H_2O_2 in algal systems

Previous research has indicated that photosynthetic H_2O_2 production is primarily a result of the Mehler reaction (i.e. pseudocyclic photophosphorylation) in algae that lack the photorespiratory pathway of higher plants (possess glycolate dehydrogenase as opposed to glycolate oxidase). In addition, the ATP formed during the Mehler reaction (without simultaneous formation of NADPH) may be an important contributor to energy requirements for carbon-concentrating mechanisms (CCM) in algal systems (Collen, et al., 1995; Sultemeyer, et al., 1993). Interestingly, Dionisio, et al. (1989) have demonstrated blue light induction of carbonic anhydrase activity in *Chlamydomonas reinhardtii*. This suggests that exposure to blue wavelengths may subsequently lead to an increase in H_2O_2 production (from increased pseudocyclic photophosphorylation to meet ATP demands of CCM). As H_2O_2 may freely diffuse across cellular membranes, catalase may play an important role in scavenging H_2O_2 that escapes ascorbate peroxidase defenses within the chloroplast and diffuses into other regions of the cell.

The subcellular localization of catalase in higher plants and algae that utilize glycolate oxidase in photorespiration has been related to phylogenetic development. In contrast, the phylogenetic relationships observed in algae do not appear to be applicable to catalase, which is localized in the microbodies regardless of the type of glycolate-oxidizing enzyme. Stabenau, et al. (1993) used sucrose gradients in order to determine the localization of several components of photorespiration in *Dunaliella* (particularly glycolate dehydrogenase). Catalase was identified as the marker enzyme for the peroxisome fraction following gradient separation, while glycolate

dehydrogenase was localized in mitochondria. The question of the importance of catalase function and subcellular localization is, as yet, unanswered for most glycolate dehydrogenase algae. Although it appears that isoform regulation and responses to a variety of light treatments may be related to the more derived characteristics of higher plants (particularly in *D. bardawil*), the physiological role of the enzyme in microalgae must first be established.

Comparing the responses of *D. bardawil* and *D. salina* suggests that *D. bardawil* may have evolved photoreceptors (blue/UV-A) and possibly phytochrome, while *D. salina* does not appear to possess these structures. Exposure to different types of spectral quality and quantity resulted in a wide variety of isozyme responses in *D. bardawil* (**Fig. 2.4**). There was no photomediated inactivation of isoforms in *D. salina* (**Fig. 2.5**), except for the inactivation of secondary isoforms following exposure to UV-A (most likely due to direct damage of the enzyme heme moiety). Other forms of *D. salina* (UTEX 1644) are known to behave metabolically much like *D. bardawil*, particularly with respect to the accumulation of β-carotene in stress conditions (Loeblich, 1982). It may be suggested that *D. salina* UTEX 1644 is a more evolved species than UTEX 200 (used in this study), and the evolution of photoreceptors has assisted in enabling the accumulation of β-carotene in this species.

Escoubas, et al. (1995) examined regulation of *cab* (chlorophyll *a,b*) gene transcription in *Dunaliella tertiolecta* as a function of light intensity. This research found that the light-dependent regulation of *cab* gene transcription is signaled by changes in the redox state of the plastoquinone pool. The ability of cells to perceive changes in light intensity are a critical factor in gene signaling for *D. tertiolecta*, as

well as a number of other algal species (Leblanc, et al., 1999; Hermsmeier, 1991). Wavelength perception has been demonstrated in other species of *Dunaliella*, as well, mostly in conjunction with the accumulation of β-carotene (Jahnke, 1999; Lers, et al., 1990). Antioxidant responses of *D. bardawil* in this study suggest the presence of specialized light receptors and their coordination with signal responses (based on the changes in isozyme activity following exposure to the different spectral treatments). In contrast, there did not appear to be any change in antioxidant responses in *D. salina* following exposure to PAR or UV radiation, indicating that there is no mechanism for specialized light perception.

UV effects on catalase isozyme activity

The effect of UV-A on catalase activity appears to be species specific and related to the inactivation or stimulation of enzyme isoforms, while the UV-B effect appears to be correlated to blue light inhibition of the enzyme, or associated photodamage. When cells were exposed to K_2CrO_4-filtered UV-B, the accumulation of β-carotene appeared to lend some selective protection from catalase inactivation in *D. bardawil* (**Fig. 2.3**). When exposed to UV-B using only the cellulose actetate filters, (the equivalent intensity of natural sunlight), catalase activity decreased significantly in all three samples tested within 24 hours of exposure. The UV-B treatment using only the cellulose acetate filter, however, allows both UV-A and UV-C wavelengths associated with the lamp emission spectrum to reach the cultures. Long-term inactivation of catalase following UV-B treatments was most likely associated with damage to photosynthesis through pigment photobleaching and subsequent radical

formation. Research has indicated that the photoinactivation of catalase occurs only in the presence of non-photosynthesizing, but not in the presence of photosynthesizing, chloroplasts (Polidoros and Scandalios, 1997). In other words, the long-term damage to catalase following exposure to high intensity UV-B is likely to be an indirect effect of photosynthetic damage and subsequent reactive oxygen formation.

Extensive research on catalase has described the loss of activity *in vitro* as dependent on the presence of oxygen, an indication that photooxidative reactions are involved (Grotjohann, et al., 1997; Polidoros and Scandalios, 1997; Feierabend and Engel; 1986). Short-term exposure to UV-B (0-3 hours; **Fig. 2.2**) resulted in significant increases in catalase activity. This finding is consistent with past research indicating that cells increase antioxidant responses as a first line of defense to environmental stress, particularly, related to light quality and quantity (Lesser, 1996a,b; Malanga and Puntarulo, 1995; Willekens, et al., 1994; Lesser and Shick, 1989).

Both PAGE analysis and measurements of enzyme activity indicate that the effects of UV-A are species specific and related to the inactivation or stimulation of the various catalase isoforms. Although the initial activity of catalase, prior to UV-A exposure was significantly, inherently greater in *D. bardawil* than *D. salina*, once exposed to UV-A, *D. bardawil* catalase activity decreased significantly (in both high and low β-carotene cultures) while *D. salina* exhibited little change in enzyme activity. PAGE analysis revealed that these results were consistent with the inactivation of both primary and secondary isoforms of catalase in *D. bardawil*

following UV-A exposure, while there was no change in the activity of the primary isoform in *D. salina* (even though secondary isoforms were inactivated).

This research indicates that catalase isozyme responses to light demonstrate a high degree of species specificity. The regulation of the various secondary isoforms in *D.bardawil* appears to be via a phytochrome response, in conjunction with a blue/UV-A photoreceptor. The inactivation of catalase isozymes is oxygen dependent, indicating that the formation of ROS is closely associated with enzyme damage (from either photooxidative reactions within the chloroplasts or associated with the heme moiety of the enzyme).

Role of accumulated β-carotene as a photoprotective shield

The accumulated β-carotene in *D. bardawil* is entirely associated with the chloroplast therefore it would be expected that only a limited amount of blue-light filtration would effect other cellular organelles. Research from Chapter I of this dissertation demonstrated that the thylakoid-accumulated β-carotene in *D. bardawil* provides a significant amount of protection to photosynthetic processes within the chloroplast following exposure to UV-A radiation through filtration of damaging blue/UV-A wavelengths. It has been established that the chloroplast is afforded significant protection, however, are the remainder of the organelles affected (protected) by the filtration of these wavelengths, as well?

Following exposure to UV-A, HL and LL cultures of *D. bardawil* exhibited a significant decrease in catalase activity, however, both responses were the same (**Fig. 2.2**). In addition, exposure to UV-B resulted in an initial increase (within 3 hours) of

catalase activity, but following 24 hours, returned to the baseline value. The cells that had accumulated massive quantities of β-carotene did not appear to have an advantage over those with low levels of β-carotene, with respect to heme inactivation and UV-A/blue light absorption (to prevent photoinactivation). Catalase isoform characteristics appear to have a high degree of species specificity within the genus *Dunaliella*, and other photosynthetic organisms. This research suggests that catalase isozymes in *Dunaliella* are regulated by a UV-A/blue light photoreceptor, possibly in conjunction with phytochrome. The inactivation of isozymes (particularly in *D. bardawil*) is oxygen dependent, indicating that the formation of reactive oxygen is related to isozyme responses, as well.

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